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Mediation of Host Immune Responses after Immunization of Neonatal Calves with a Heat-Killed *Mycobacterium avium* subsp. *paratuberculosis* Vaccine[▽]

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A major drawback of current whole-cell vaccines for *Mycobacterium avium* subsp. *paratuberculosis* is the interference with diagnostic tests for bovine tuberculosis (TB) and paratuberculosis. The current study was designed to explore the effects of immunization with a heat-killed whole-cell vaccine (Mycopar) on diagnostic test performance and to characterize host immune responses to vaccination over a 12-month period. Neonatal dairy calves were assigned to treatment groups consisting of (i) controls, not vaccinated ($n = 5$), and (ii) vaccinates, vaccinated with Mycopar vaccine ($n = 5$). The results from this study demonstrated a rapid initiation of *M. avium* subsp. *paratuberculosis*-specific gamma interferon (IFN- γ) in vaccinated calves by 7 days, with robust responses throughout the study. Vaccinated calves also had responses to *M. bovis* purified protein derivative tuberculin (BoPPD) but minimal reactivity to ESAT-6/CFP-10, an *M. bovis* recombinant fusion protein. The levels of antigen-specific interleukin-4 (IL-4) and IL-10 were markedly decreased in vaccinated calves between days 7 and 90 of the study but thereafter were similar to the levels in controls. Vaccinated calves began to seroconvert at 4 months, with 4/5 calves having detectable *M. avium* subsp. *paratuberculosis* antibody by 6 months. The responses in test platforms for bovine TB were negligible in the vaccine group, as only one calf had a response, which was in the suspect range of the comparative cervical skin test. Serum antibody responses to *M. bovis* antigens ESAT-6, CFP-10, and MPB83 were negative on the Vet TB STAT-PAK, DPP VetTB, and DPP BovidTB tests. These results suggest that the Mycopar vaccine will interfere with diagnostic tools for paratuberculosis but result in low interference with the comparative cervical skin test and emerging serologic tests for *M. bovis*.

Vaccination has proven to be effective in reducing the *Mycobacterium avium* subsp. *paratuberculosis* tissue burden in infected animals (28). It has also been shown to be effective in reducing fecal shedding, thereby controlling the spread of disease within and between herds (14). Vaccination also alleviates signs of clinical disease, so it is helpful in allaying losses associated with reproductive problems and reduced milk production (13, 32). One major disadvantage to using a whole-cell vaccine is the inability of standard serologic diagnostic tests for bovine paratuberculosis to distinguish vaccinates from *M. avium* subsp. *paratuberculosis*-infected animals (8, 19). It has been shown that whole-cell vaccines for *M. avium* subsp. *paratuberculosis* will trigger both Th1- and Th2-mediated immune responses, as demonstrated by antigen-specific gamma interferon (IFN- γ) responses and *M. avium* subsp. *paratuberculosis*-specific serum antibody, thereby confounding their use as diagnostic tools for the detection of *M. avium* subsp. *paratuberculosis* infection (9, 17, 19). However, it is possible that other immunologic parameters may be useful as screening tools that can differentiate *M. avium* subsp. *paratuberculosis* infection from vaccination. Recent studies have shown that vaccination with whole-cell vaccines will elicit increases in CD4

and $\gamma\delta$ T cells and CD4 CD25 T cell populations in recall to antigen stimulation (9, 22). A more thorough assessment of host immune responses to vaccination will provide us with information about protective correlates associated with reduced clinical disease.

Another major concern for the use of paratuberculosis vaccines is the potential interference with current diagnostics for bovine tuberculosis (TB). The caudal fold skin test (CFT) is the most common tuberculin test and is widely used as an initial screening tool for bovine tuberculosis in the field (10). The CFT is based upon a response to a singular antigen, *M. bovis* purified protein derivative tuberculin (BoPPD). However, the use of the comparative cervical test (CCT) is recommended as a complementary test to the CFT as it uses a dual antigen approach (bovine and avian PPDs) to discriminate between true *M. bovis* infection and reactivity due to environmental mycobacteria, including *M. avium* subspecies. Paratuberculosis infection has been shown to have a negative effect on the interpretation of the tuberculin skin test, with elevated responses to BoPPD compared to those in noninfected cattle, but little work has been done to evaluate the effects of paratuberculosis vaccination on the bovine tuberculin test (1). New serologic tests for the detection of *M. bovis* infection have recently been developed and are demonstrating high levels of sensitivity and specificity in the detection of bovine tuberculosis (18). However, there are no available data to determine whether these new serologic test platforms will reduce cross-reactivity with *M. avium* subsp. *paratuberculosis* antigens asso-

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ciated with the paratuberculosis vaccine. The proposed research will explore the cross-reactivity of the current U.S. heat-killed whole-cell vaccine for *M. avium* subsp. *paratuberculosis* with new serologic diagnostic tools for bovine TB.

MATERIALS AND METHODS

Animals. Neonatal Holstein dairy calves were obtained from herds in Iowa and Minnesota at 1 to 2 days of age. The farms were status level 4 herds enrolled in the Voluntary Bovine Johne's Control Program, with no reportable incidence of Johne's disease in the last 4 to 5 years and a 99% probability that they were free of paratuberculosis. Calves were housed in biosafety level 2 containment barns for the duration of the study. Calves were allowed to acclimate to their environment for 1 week prior to the initiation of the study. Standard commercial milk replacer (Land O Lakes, Shoreview, MN) was fed 2× per day at 10-h intervals during the first 6 weeks of age, and then calves were weaned onto calf starter (Kent Feeds, Muscatine, IA) and gradually switched over to a mixed pelleted ration for the remainder of the study. The treatment groups consisted of (i) controls, not vaccinated ($n = 5$), and (ii) vaccinates, vaccinated with Mycopar vaccine ($n = 5$). Blood and fecal samples were collected on 2 consecutive days prior to the initiation of the study (days -2 and 0) and averaged for prevaccination measurements. Calves were then vaccinated subcutaneously in the dewlap-brisket area according to standard procedure with a 0.5-ml dose with the wild-type commercial vaccine consisting of a heat-killed whole-cell suspension of *M. avium* subsp. *paratuberculosis* in oil (Mycopar, Fort Dodge Animal Health, Fort Dodge, IA). Calves were sampled throughout the study on days 7, 14, and 28 and at 3, 6, 9, and 12 months. All procedures performed on the animals were approved by the Institutional Animal Care and Use Committee (National Animal Disease Center [NADC], Ames, IA).

Blood collection and culture conditions. Peripheral blood mononuclear cells (PBMCs) were isolated from the buffy coat fractions of blood. PBMCs were resuspended in complete medium (RPMI-1640 [Gibco, Grand Island, NY] with 10% fetal calf serum [Atlanta Biologicals, Atlanta, GA], 100 U of penicillin G sodium [Gibco] per ml, 100 µg of streptomycin sulfate [Gibco] per ml, 0.25 µg of amphotericin B [Gibco] per ml, and 2 mM *L*-glutamine [Gibco]). Cells were cultured at 2.0×10^6 /ml in replicate 48-well flat-bottom plates (Corning Incorporated, Corning, NY) at 39°C in 5% CO₂ in a humidified atmosphere. Duplicate wells were set up for each animal for each *in vitro* treatment. *In vitro* treatments consisted of no stimulation (medium only), 10 µg/ml concanavalin A (ConA; Sigma Chemical Co., St. Louis, MO), 10 µg/ml pokeweed mitogen (PWM; Sigma), 10 µg/ml johnin purified protein derivative (JPPD; National Veterinary Services Laboratory, Ames, IA), 10 µg/ml *M. bovis* purified protein derivative (BoPPD; NVSL), 1 µg/ml recombinant ESAT-6/CFP-10 fusion protein (rEC) from *M. bovis* (provided by F. C. Minion, Iowa State University), and 10 µg/ml of a whole-cell sonicate of strain K-10 of *M. avium* subsp. *paratuberculosis* (MPS). The MPS was prepared by sonication of *M. avium* subsp. *paratuberculosis* (1×10^9 /ml) in phosphate-buffered saline (PBS) at 25 W for 25 min on ice (Tekmar sonic disturber; Tekmar, Lorton, VA), and the protein concentration was determined as previously described (26). After 24 h, one set of plates were removed and centrifuged at $400 \times g$ for 5 min. Supernatants were removed without disturbing the cells in culture and stored at -20°C prior to cytokine measurement. The replicate set of plates was incubated for 6 days, and cells were harvested for flow cytometric analyses.

Cytokine analyses of cell culture supernatants. Bovine IFN-γ was measured by using the Bovigam test kit (Prionics, La Vista, NE) as described by the manufacturer. A standard curve was generated using recombinant bovine IFN-γ (3.12 to 100 ng/ml; Thermo Scientific, Rockford, IL). Bovine interleukin-10 (IL-10) was quantified by coating MaxiSorp microtiter plates (Nunc, Rochester, NY) with mouse anti-bovine IL-10 in coating buffer (0.05 M carbonate-bicarbonate buffer, pH 9.6 [MCA2110], 2 µg/ml; Serotec, Raleigh, NC) overnight at room temperature (RT). After washing 5 times with PBS containing 1% Tween 80, samples and dilutions of bovine IL-10 standard (0.3125 to 20 ng/ml) (generous gift from Jayne Hope, Compton, United Kingdom) were added to duplicate wells and incubated at RT for 1 h. After being washed, plates were incubated with mouse anti-bovine IL-10-biotin (MCA2111B; Serotec) for 1 h. Plates were washed again and incubated with avidin-horseradish peroxidase (HRP) conjugate (1:800) (PharMingen, San Diego, CA) for 45 min at RT. After another wash cycle, plates were incubated with substrate solution (40 mM ABTS [2,2'-azino-diethylbenzothiazoline-6-sulfonic acid]; US Biological, Swampscott, MA). Color development was quantified after 30 min by measuring absorbance at 405 nm with a Wallac Victor 1420 multilabel counter enzyme-linked immunosorbent assay (ELISA) plate reader (Perkin-Elmer, Gaithersburg, MD). To quantify

TABLE 1. Primary antibodies^a

Antigen	MAb clone	Isotype	Working MAb concn (µg/ml) ^b	Specificity
CD4	GC50A1	IgM	14	T-helper cell
CD5	B29a	IgG _{2a}	7	Activation marker
CD8	BAQ111A	IgM	14	T-cytotoxic/suppressor cell
CD25	CACT116A	IgG1	15	IL-2 receptor
	CACT108A	IgG _{2a}	15	
	LCTB2A	IgG ₃	15	
CD26	CACT114A	IgG _{2b}	15	Activation marker
CD45RO	GC42A1	IgG ₁	10	Memory/activation marker
N12	CACT61A	IgM	14	γδ-cell receptor
B cell	BAQ155	IgG1	7	Total B cell

^a Obtained from VMRD, Inc. (Pullman, WA). MAb, monoclonal antibody.

^b Diluted in PBS with 1% fetal calf serum and 0.04% sodium azide.

bovine IL-4 in culture supernatants, plates were coated with mouse anti-bovine IL-4 (clone CC313, 5 µg/ml; Serotec) in 0.05 M carbonate-bicarbonate buffer and incubated overnight at 4°C. After being washed, plates were blocked with PBS containing 1% bovine serum albumin (BSA) and 0.05% Tween 20 for 1 h at RT. After being washed, samples and dilutions of the standard (recombinant bovine IL-4 [PBP006], 15.6 to 2,000 pg/ml; Serotec) were incubated for 1 h at RT, followed by incubation with mouse anti-bovine IL-4-biotin antibody (clone CC314, 2.5 µg/ml; Serotec) for 1 h at RT, streptavidin-horseradish peroxidase (1:1,000; GE Healthcare, Piscataway, NJ) for 45 min at RT, and then ABTS substrate (US Biological) for 1 h at RT, followed by measuring absorbance at 405 nm as described above. Bovine IL-12 was measured by ELISA after coating plates with mouse anti-bovine IL-12 antibody (MCA1782EL, 8 µg/ml; Serotec) diluted in 0.05 M carbonate-bicarbonate buffer overnight at 4°C. Plates were then blocked with 1% BSA overnight at 4°C, followed by the addition of samples and standard dilutions (55 to 1,500 U/ml; kind gift from Jayne Hope, Compton, United Kingdom), and were incubated for 1 h at RT. This was followed by incubation with biotinylated anti-bovine IL-12 (MCA2173B, 1 µg/ml; Serotec) for 1 h at RT, incubation with streptavidin-horseradish peroxidase (1:800) and ABTS substrate for 1 h at RT, and then measurement of absorbance at 405 nm as described above.

Flow cytometric analysis. Briefly, 6-day culture plates were centrifuged at 1,500 rpm for 5 min and the supernatant was decanted. Cells were gently resuspended in 300 µl of PBS (0.15 M, pH 7.4). In 96-well round-bottom plates (Corning Incorporated, Corning, NY), 50-µl amounts of the cell suspension were added to wells containing 50 µl of primary monoclonal antibody to CD4, CD8, and γδ T cells, B cells, CD25, CD26, CD5, and CD45RO (Table 1). All wells received 10 µg/ml of DAPI (4'-6-diamidino-2-phenylindole; Sigma) to differentiate live from dead cells and allow gating on viable cells. Cells were then incubated at 4°C for 30 min. After incubation, plates were centrifuged at 1,250 rpm for 2 min at 4°C and the supernatant decanted. Amounts of 100 µl of secondary antibody cocktail consisting of fluorescein-conjugated anti-mouse IgM (Southern Biotech, Birmingham, AL), R-phycoerythrin-conjugated goat F(ab)₂ anti-mouse IgG_{2a} (Southern Biotech, Birmingham, AL), and peridinin-chlorophyll-protein complex-conjugated rat anti-mouse IgG1 (Becton Dickinson, San Jose, CA) diluted 1:312, 1:625, and 1:42, respectively, in PBS with 1% fetal calf serum and 0.04% sodium azide were then added to designated wells, and the plate was centrifuged again at 1,250 rpm for 2 min at 4°C. The cells were then resuspended in 200 µl of BD FACS lyse (BD Biosciences, San Jose, CA) for immediate flow cytometric analysis. Samples were evaluated using 30,000 events per sample using a FACScan flow cytometer (CellQuest Software; Becton Dickinson). Mononuclear cells, based on forward- and side-scatter characteristics, were analyzed for cell surface marker expression (FlowJo; Tree Star, Inc., San Carlos, CA).

Serologic assays. Serum was harvested from whole blood and assayed for the presence of *M. avium* subsp. *paratuberculosis* antibodies by commercial ELISA (Herdchek) and Western blotting. Electrophoresis and immunoblot assays were performed using previously reported procedures (2). Briefly, the reactivities of serum samples from calves against MPS (whole-cell sonicate of *M. avium* subsp. *paratuberculosis* strain K-10) were assessed using the Mini-Protein II slot blot device (Bio-Rad, Richmond, California). Antigen was electrophoresed through preparative 12% (wt/vol) polyacrylamide gels and transferred to nitrocellulose

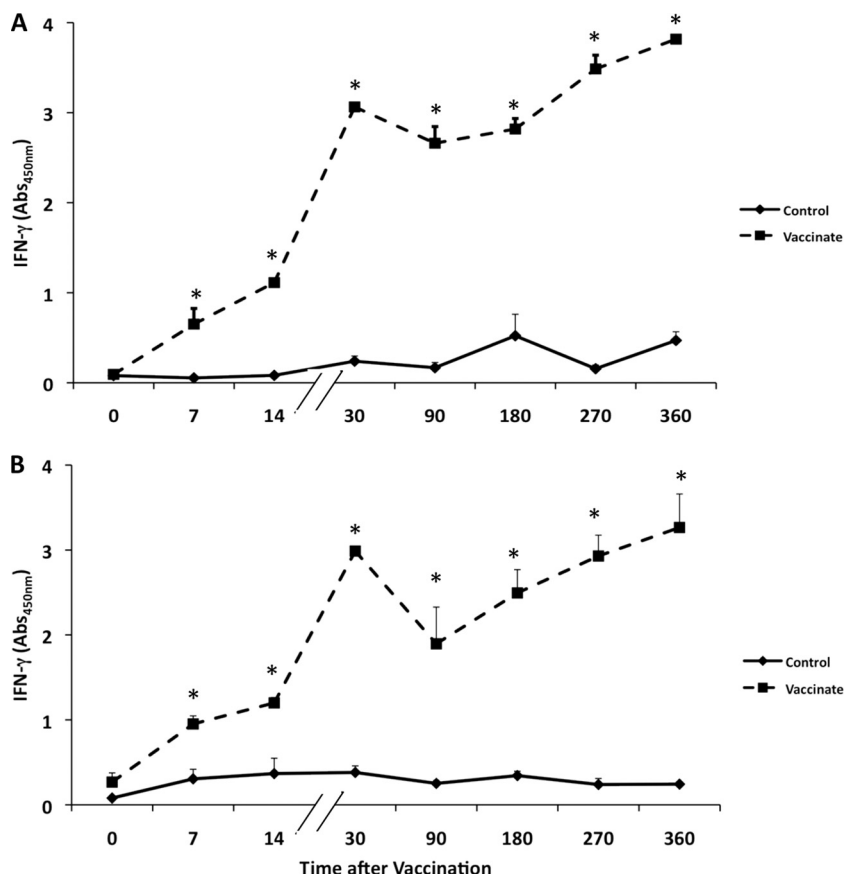


FIG. 1. Secretion of IFN- γ (ng/ml) by PBMCs of control calves and calves vaccinated with a heat-killed whole-cell *Mycobacterium avium* subsp. *paratuberculosis* vaccine at various time points during the 12-month study when stimulated with johnin purified protein derivative (JPPD) (A) and with whole-cell sonicate of *Mycobacterium avium* subsp. *paratuberculosis* (MPS) (B). Data are expressed as means \pm SEMs. Significant differences between treatment group within given time points are represented by asterisks (*, $P < 0.01$; **, $P < 0.05$). Abs, absorbance.

filters. These filters were placed in a blocking solution consisting of phosphate-buffered saline plus 0.1% Tween 20 (PBST) and 2% (wt/vol) bovine serum albumin (PBST-BSA). After the blocking, the filters were placed into the slot blot device and individual sera, diluted 1:200 in PBST-BSA, were added to independent slots. After a 2-h incubation with gentle rocking, the blots were washed three times with PBST and incubated with horseradish peroxidase-conjugated anti-goat IgG heavy and light chains (Vector Laboratories, Burlingame, CA) diluted 1:20,000 in PBST-BSA for 1.5 h. The blots were again washed three times with PBST and developed for chemiluminescence in SuperSignal detection reagent (Thermo Scientific, Rockford, IL).

For detection of *M. bovis*-specific response, the VetTB STAT-PAK assay, the dual-path platform (DPP) VetTB assay, and the DPP BovidTB assay (Chembio Diagnostic Systems, Inc., Medford, NY) were performed as described previously (11). These immunochromatographic tests use *M. bovis* antigens, such as ESAT-6, CFP10, and MPB83, to measure *M. bovis* antibody (11, 12, 18). The tests require minimal amounts of serum sample (5 to 30 μ l), and results are available within 20 min, by visually monitoring the appearance of bands in the reaction zone. The use of an optical reader to monitor these tests is optional and in the present study was unnecessary due to the negative results for vaccinated calves. All sera were coded and tested blindly.

Skin test. The comparative cervical skin test was performed at 6 months both as a diagnostic tool and to determine the *in vivo* cell-mediated response to vaccination. Skin tests were performed as specified in the "Uniform Methods and Rules for Bovine Tuberculosis Eradication" circular (APHIS 91-45-011) of the USDA Animal and Plant Health Inspection Service (30), with minor modifications. Briefly, skin thickness was measured with calipers immediately prior to and 72 h after injection of *M. avium* purified protein derivative (AvPPD) and *M. bovis* purified protein derivative (BoPPD) to the midcervical region of each animal. Data are presented as the change in skin thickness (mm) from preinjection measurements (mean \pm standard error of the mean [SEM]) for each PPD.

Statistical analyses. Data were analyzed by repeated measures using the PROC MIXED procedure of the Statistical Analysis System (SAS Institute, Inc., Cary, NC). The model included the fixed effects of treatment (vaccination), day (time after vaccination), and the treatment-times-day interaction, the random effect of calves nested within treatment, and the residual error. When significant effects ($P < 0.05$) due to treatment, day, or treatment-times-day interactions were detected, means separation was conducted by using the Tukey-Kramer option in SAS.

RESULTS

Vaccination with Mycopar resulted in highly significant ($P < 0.01$) upregulation of IFN- γ production by PBMCs in response to either of the *M. avium* subsp. *paratuberculosis* antigen preparations (JPPD or MPS) beginning at 7 days postvaccination (Fig. 1A and B). Antigen-specific IFN- γ continued to increase in a linear fashion up to 30 days and then began to level off. At 6 months (180 days), all calves were administered a comparative cervical skin test, resulting in a boost in IFN- γ secretion that lasted throughout the remainder of the study (360 days). An evaluation of IFN- γ responses to nonspecific *in vitro* stimulants (ConA and PWM), as well as *M. bovis* antigen preparations (BoPPD and rEC), at 1 and 12 months postvaccination is presented in Fig. 2. As shown in Fig. 1, both JPPD and MPS evoked robust responses in vaccinated calves at both time points. Similar responses in IFN- γ secretion to BoPPD ($P <$

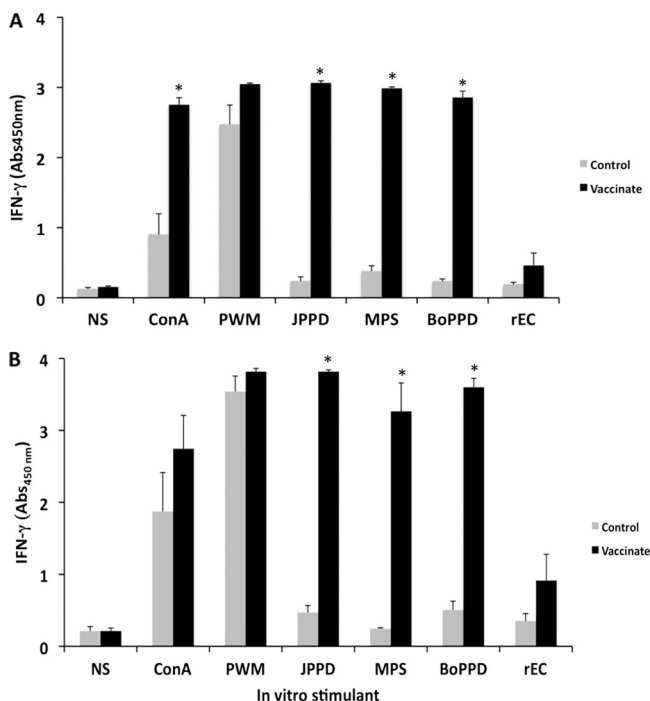


FIG. 2. Comparison of *in vitro* stimulation with medium control (NS [not stimulated]), concanavalin A (ConA), pokeweed mitogen (PWM), johnin purified protein derivative (JPPD), whole-cell sonicate of *Mycobacterium avium* subsp. *paratuberculosis* (MPS), bovine purified protein derivative (BoPPD), and recombinant ESAT-6/CFP10 fusion protein (rEC) on IFN- γ (ng/ml) secretion by PBMCs isolated from control calves and calves vaccinated with a heat-killed whole-cell *Mycobacterium avium* subsp. *paratuberculosis* vaccine at 1 month of infection (A) and 12 months postvaccination (B). Data are expressed as means \pm SEMs. Significant differences between treatment groups are represented by asterisks (*, $P < 0.01$; **, $P < 0.05$). Abs, absorbance.

0.01) but not to the rEC protein preparation were noted in vaccinated calves (Fig. 2A and B). Interestingly, treatment differences were also noted after ConA stimulation of cells, with higher ($P < 0.01$) levels of IFN- γ for vaccinated calves at 1 month (Fig. 2A) but not at 12 months (Fig. 2B).

An overall trend ($P = 0.06$) for effects due to treatment was demonstrated in IL-4 secretion, with minimal effects noted due to stimulation of cells *in vitro*. After collapsing data from all time points within an *in vitro* group, as depicted in Fig. 3, an upregulation in IL-4 secretion by ConA-stimulated cells and a downregulation in cells stimulated with MPS (overall significance $P < 0.02$) in vaccinated calves were noted. Increases in antigen-mediated IL-4 secretion occurred at 7 and 14 days for control calves but not vaccinated calves (Fig. 4A). This effect was consistent after stimulation of cells with either of the *M. avium* subsp. *paratuberculosis* antigen preparations (JPPD or MPS) (data not shown) but was not observed for cells stimulated with BoPPD or rEC. Interestingly, a similar upregulation ($P < 0.05$) of IL-10 in MPS-stimulated cell cultures was observed between 7 and 90 days of the study for control calves (Fig. 4B). There were no discernible effects due to vaccination or *in vitro* stimulation of cells on the secretion of IL-12 (data not shown).

Additional immunologic responses to vaccination were char-

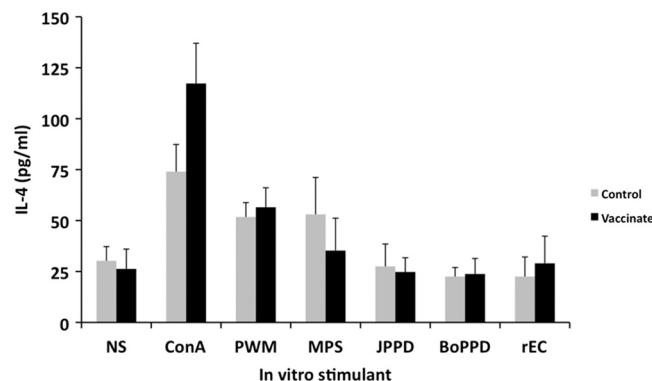


FIG. 3. Comparison of *in vitro* stimulation with medium control (NS), concanavalin A (ConA), pokeweed mitogen (PWM), johnin purified protein derivative (JPPD), whole-cell sonicate of *Mycobacterium avium* subsp. *paratuberculosis* (MPS), bovine purified protein derivative (BoPPD), and recombinant ESAT-6/CFP10 fusion protein (rEC) on IL-4 (pg/ml) secretion by PBMCs isolated from control calves and calves vaccinated with a heat-killed whole-cell *Mycobacterium avium* subsp. *paratuberculosis* vaccine. Data are expressed as means \pm SEMs. Significant differences between treatment groups are represented by asterisks (*, $P < 0.01$; **, $P < 0.05$).

acterized by flow cytometric analyses of immune cell subpopulations. Vaccination did not appear to affect CD8 or $\gamma\delta$ populations, regardless of the *in vitro* stimulant used (data not shown). However, the percentage of total CD4 T cells in PBMCs stimulated with JPPD was significantly ($P < 0.01$) increased for vaccinated calves within 1 month after vaccina-

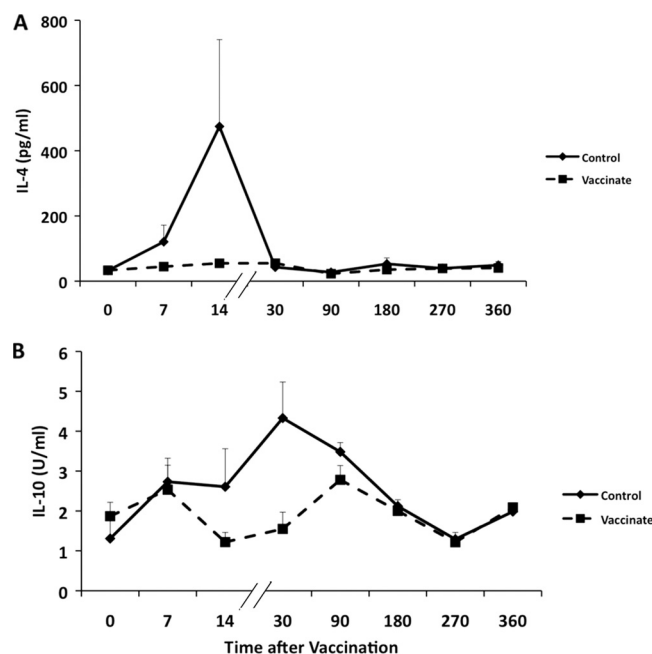


FIG. 4. Secretion of IL-4 (pg/ml) and IL-10 (U/ml) by PBMCs of control calves and calves vaccinated with a heat-killed whole-cell *Mycobacterium avium* subsp. *paratuberculosis* vaccine when stimulated with johnin purified protein derivative (JPPD) at various time points during the 12-month study. Data are expressed as means \pm SEMs. Significant differences between treatment groups are represented by asterisks (*, $P < 0.01$; **, $P < 0.05$).

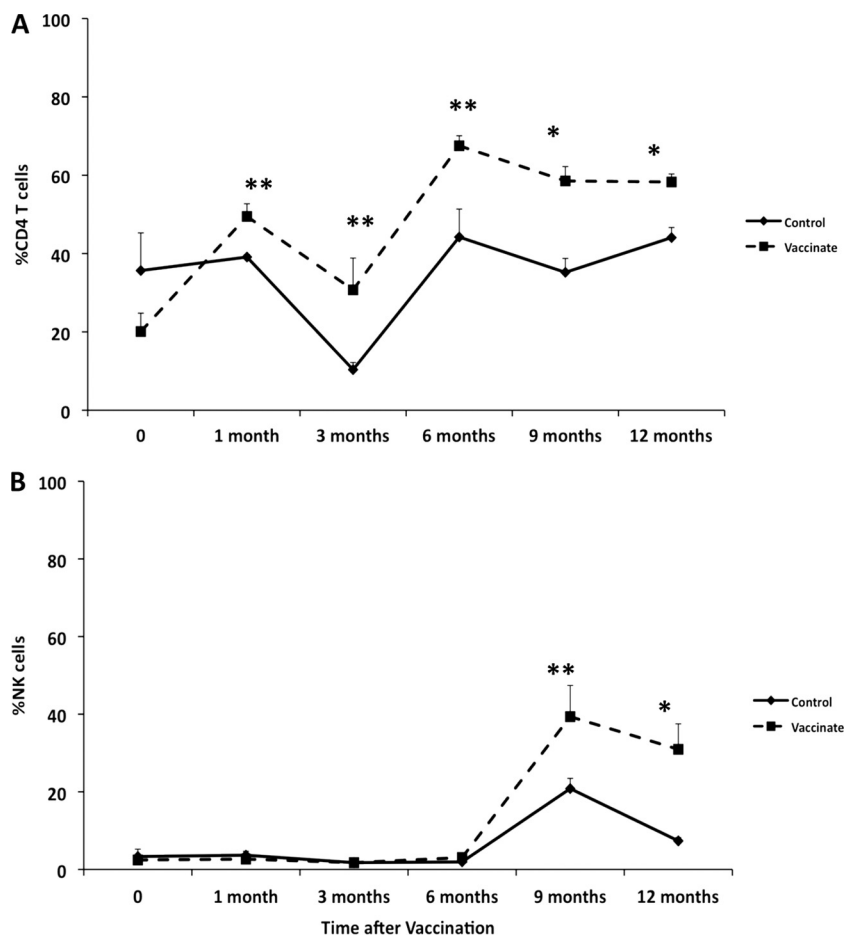


FIG. 5. Percentages of CD4 T cells (A) and NK (B) cells within total PBMCs of control calves and calves vaccinated with a heat-killed whole-cell *Mycobacterium avium* subsp. *paratuberculosis* vaccine when stimulated with johnin purified protein derivative (JPPD) at various time points during the 12-month study. Data are expressed as means \pm SEMs. Significant differences between treatment groups within given time points are represented by asterisks (*, $P < 0.01$; **, $P < 0.05$).

tion (Fig. 5A). A delayed effect of vaccination was observed on natural killer (NK) cells (CD335⁺ T cells), which were upregulated at 9 and 12 months in PBMCs stimulated *in vitro* with *M. avium* subsp. *paratuberculosis* antigens (Fig. 5B; data for JPPD are shown). Some increases in the percentage of NK cells in response to BoPPD were also noted at these time points in vaccinated calves, but no increases were observed for rEC (data not shown).

Vaccination did result in increased expression of T cell activation markers on some immune cell subpopulations. An upregulation of CD25 expression was observed on T cells stimulated with JPPD within 3 months postvaccination and continued to increase throughout the study (Fig. 6). In contrast, a marked ($P < 0.01$) increase in antigen-specific CD25 expression was noted within the CD4⁺ subpopulation at 1 month, regardless of vaccination status (Fig. 6B). By 3 months, CD4 CD25 expression had begun to decline for vaccinated calves but was still upregulated in control animals. At later time points (6, 9, and 12 months) of the study, CD4 CD25 expression was significantly ($P < 0.05$) higher for vaccinated calves than for the nonvaccinated controls, in a pattern similar to the results for the total T cell population. Perhaps more striking

were the results of CD26 activation marker expression on T cells (Fig. 7A and B). The upregulation of antigen-specific CD26 expression on total T cells was increased ($P < 0.05$) within 1 month and extended throughout the 12-month study for vaccinated calves (Fig. 7A). Similarly, within 3 months, CD26 expression on CD4⁺ T cells was highly upregulated ($P < 0.05$) in vaccinated calves. (Fig. 7B). Although CD26 expression declined between 3 and 12 months, it was consistently higher for vaccinated calves. Neither the total B cell populations nor the B cell subpopulations defined by CD5 and CD45RO expression were affected when stimulated *in vitro* using PWM, a polyclonal activator (Fig. 8). However, vaccination did result in a decrease ($P < 0.05$) in the percentage of B cells following stimulation of PBMCs with either JPPD or MPS at 12 months (Fig. 8). Interestingly, an upregulation in CD5 and CD45RO expression on B cells was also noted after antigen stimulation of cells *in vitro*; however, this effect was only significant ($P < 0.01$) for MPS.

The temporal appearance of *M. avium* subsp. *paratuberculosis*-specific antibody after vaccination was measured using a commercial ELISA (Herdchek) and immunoblot. Four of 5 vaccinated calves seroconverted during the 12-month study

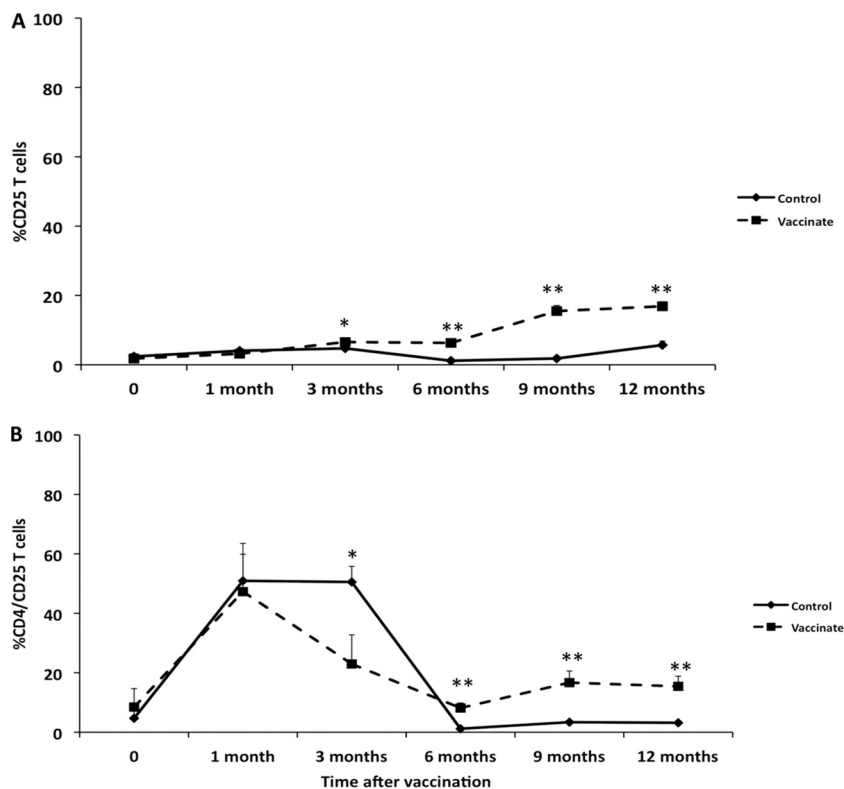


FIG. 6. Percentages of total CD25 T cells (A) and CD4 CD25 T cells (B) within total PBMCs of control calves and calves vaccinated with a heat-killed whole-cell *Mycobacterium avium* subsp. *paratuberculosis* vaccine when stimulated with johnin purified protein derivative (JPPD) at various time points during the 12-month study. Data are expressed as means \pm SEMs. Significant differences between treatment groups within given time points are represented by asterisks (*, $P < 0.01$; **, $P < 0.05$).

period, as demonstrated in Fig. 9. Serum antibody was measurable in some vaccinated calves by 3 months postvaccination, and at 4 months, all calves that eventually seroconverted were positive. Antibody titers (sample-to-positive ratio) continued to increase in vaccinated calves throughout the study period. Immunoblot analyses for calf sera across all time points (0, 7, 14, and 28 days and 3, 6, 9, and 12 months) was performed using a whole-cell sonicate preparation of *M. avium* subsp. *paratuberculosis* strain K-10 as the antigen to assess the presence of serum antibodies. Sample blots for a representative calf from each treatment group are shown (Fig. 10). There was a distinct difference in the banding pattern due to vaccination of calves. Banding on blots for control animals was minimal and occurred between 40 and 50 kDa. In contrast, vaccination of calves resulted in the appearance of multiple bands ranging between 20 and 75 kDa. A good portion of these bands appeared between months 3 and 6 of the study, indicative of a gradual acquisition of antibody upon exposure to the vaccine. This banding pattern and the time of appearance were consistent among all vaccinated calves except for the one calf that failed to demonstrate seroconversion with the commercial ELISA.

We further defined the effects of vaccination on standard and novel diagnostic tests for *M. bovis* in cattle to determine whether use of the vaccine to control *M. avium* subsp. *paratuberculosis* infections will lead to false-positive TB diagnoses. The effect of vaccination on the results of a comparative cer-

vical skin test measuring reactivity to *M. avium* PPD and *M. bovis* PPD is presented in Fig. 11. Only the vaccinated calves demonstrated any reactivity in the skin test, with significantly higher ($P < 0.01$) increases in skinfold thickness noted for both AvPPD and BoPPD compared to the results for the control calves (Fig. 11). The reactions to AvPPD (18.9 ± 5.9) were significantly ($P < 0.01$) higher than the BoPPD reactions (6.9 ± 0.9) in vaccinated calves. Although BoPPD reactivity was present for vaccinated calves, only one calf fell into the suspect category, and all other calves were deemed nonreactors (Fig. 12). In addition, all serum reactions to *M. bovis* proteins (ESAT-6, CFP-10, and MPB83) using the novel serologic tests VetTB STAT-PAK, DPP VetTB, and DPP BovidTB were negative (data not shown), with the exception of one animal in the vaccinate treatment group that was weakly positive on the VetTB STAT-PAK test.

DISCUSSION

Vaccination has been a successful tool for the management of many infectious diseases in cattle and other ruminants, and paratuberculosis is no exception. Paratuberculosis vaccine studies have demonstrated the induction of both cellular and humoral immune responses; however, it is widely accepted that vaccination will not prevent infection (3, 22, 28). The key benefits to vaccination are reduced fecal shedding of *M. avium* subsp. *paratuberculosis* (28) and reduced clinical signs in in-

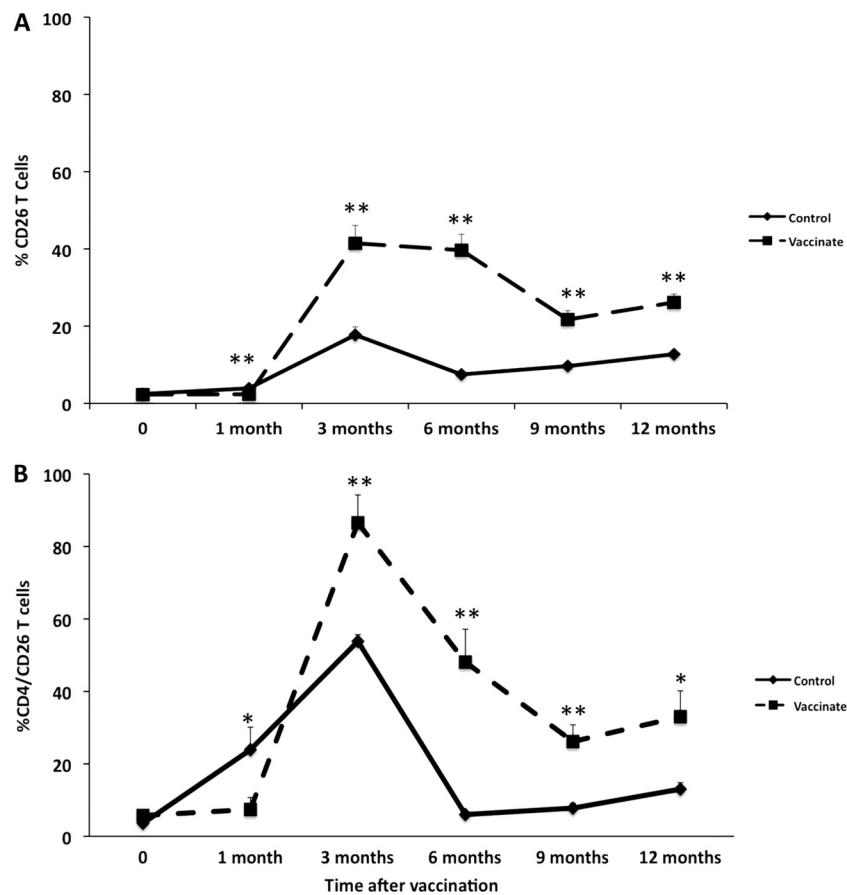


FIG. 7. Percentages of total CD26 T cells (A) and CD4CD26 T cells (B) cells within total PBMCs of control calves and calves vaccinated with a heat-killed whole-cell *Mycobacterium avium* subsp. *paratuberculosis* vaccine when stimulated with johnin purified protein derivative (JPPD) at various time points during the 12-month study. Data are expressed as means \pm SEMs. Significant differences between treatment groups within given time points are represented by asterisks (*, $P < 0.01$; **, $P < 0.05$).

fectured animals, with evidence suggesting a reduction in the incidence of disease within herds (14, 23). Although vaccination of ruminants is widely used in Europe, the Middle East, and Australia, less than 10% of dairy operations in the United

States currently vaccinate for Johne's disease (31). The rising incidence of Johne's disease in U.S. dairy herds in recent years is forcing producers to consider vaccination more carefully as an additional management tool to prevent the spread of infec-

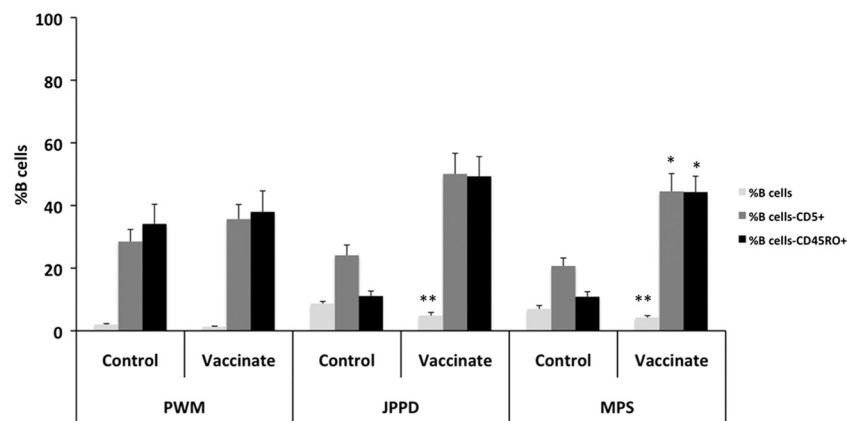


FIG. 8. Percentages of total B cells, CD5⁺ B cells/total B cells, and CD45 RO⁺ B cells/total B cells within total PBMCs of control calves and calves vaccinated with a heat-killed whole-cell *Mycobacterium avium* subsp. *paratuberculosis* vaccine when stimulated with pokeweed mitogen (PWM), johnin purified protein derivative (JPPD), and whole-cell sonicate of *Mycobacterium avium* subsp. *paratuberculosis* (MPS) at 12 months postvaccination. Data are expressed as means \pm SEMs. Significant differences between treatment groups are represented by asterisks (*, $P < 0.01$; **, $P < 0.05$).

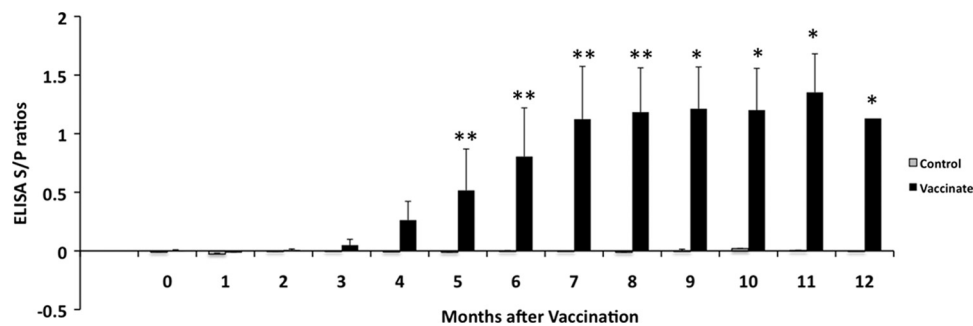


FIG. 9. Serum antibodies to *Mycobacterium avium* subsp. *paratuberculosis* in control calves and calves vaccinated with a heat-killed whole-cell *Mycobacterium avium* subsp. *paratuberculosis* vaccine measured by commercial ELISA during the 12-month study. Data are expressed as means \pm SEMs. Significant differences between treatment groups within given time points are represented by asterisks (*, $P < 0.01$; **, $P < 0.05$). S/P ratios, sample-to-positive ratios.

tion. Control of intracellular infections such as *M. avium* subsp. *paratuberculosis* is generally thought to be the result of Th1-mediated immunity in the host (24), yet vaccination invokes both cell-mediated and humoral immune responses (3, 9). With this in mind, we must consider the utility of current immune-based diagnostic tools for the detection of Johne's disease in vaccinated herds. A major impediment to the use of vaccination is the potential to result in false-positive interpretation of diagnostic tests for paratuberculosis and bovine tu-

berculosis. With the reemergence of bovine tuberculosis in U.S. dairy herds, it is critical that a clear interpretation of infection can be made in herds vaccinated for *M. avium* subsp. *paratuberculosis*.

The present study demonstrated that vaccination with the Mycopar vaccine elicited strong IFN- γ responses to *M. avium* subsp. *paratuberculosis* antigens (JPPD and MPS) within 7 days, an effect that was maintained throughout the 12-month study. This is in agreement with the results of studies evaluating other whole-cell vaccines (Neoparasec and Gudair) for Johne's disease and is suggestive of a protective Th1-mediated immune response in the host. Similar induction of antigen-specific IFN- γ responses has been demonstrated in calves in the early stages of experimental infection with *M. avium* subsp. *paratuberculosis*, indicating that exposure to *M. avium* subsp. *paratuberculosis* in live or killed form will elicit strong IFN- γ responses (27). The use of BoPPD as an antigen in the IFN- γ assay resulted in responses that mimicked those of *M. avium* subsp. *paratuberculosis* antigens, including both the robustness and the persistence of the responses. Parallel reactivities to JPPD and BoPPD in the IFN- γ test had previously been shown in long-term assessment of cattle herds administered a heat-killed vaccine (19). Although the reactivity to JPPD was stron-

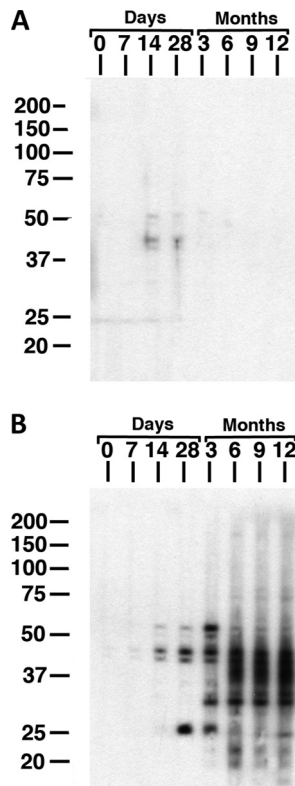


FIG. 10. Immunoblot of sera from a representative calf in each treatment group. (A) Control. (B) Vaccinate. Sera were blotted against the whole-cell sonicate preparation of *Mycobacterium avium* subsp. *paratuberculosis* strain K-10. Lane markings represent days 0, 7, 14, and 28 and the 3-, 6-, 9-, and 12-month sampling times of the study. Molecular sizes (kDa) are indicated on the left.

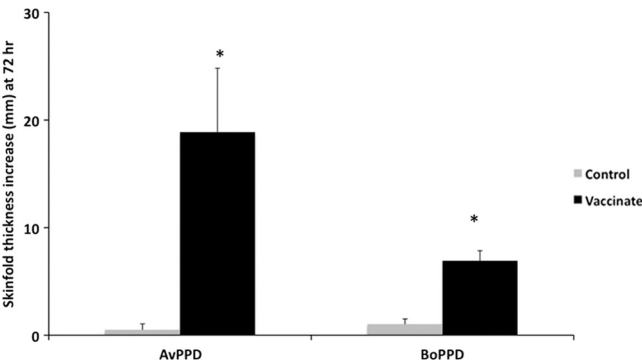


FIG. 11. Skinfold thickness increases in response to *M. avium* purified protein derivative (AvPPD) and *M. bovis* purified protein derivative (BoPPD) in control calves and calves vaccinated with a heat-killed whole-cell *Mycobacterium avium* subsp. *paratuberculosis* vaccine at 6 months postvaccination. Data are expressed as means \pm SEMs. Significant differences between treatment groups within given time points are represented by asterisks (*, $P < 0.01$; **, $P < 0.05$).

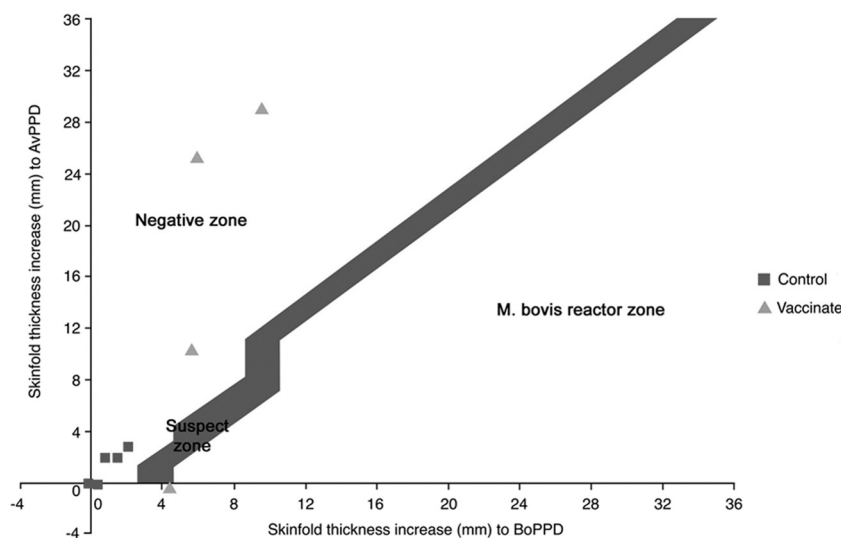


FIG. 12. Scattergram demonstrating skinfold thickness increases (mm) in response to *M. avium* purified protein derivative (AvPPD) and *M. bovis* purified protein derivative (BoPPD) in control calves and calves vaccinated with a heat-killed whole-cell *Mycobacterium avium* subsp. *paratuberculosis* vaccine. The gray zone within the diagram indicates a suspect zone determined by skinfold increase to AvPPD and BoPPD in cattle and bison. If data points fall to the left or right of the gray zone, animals are considered negative (nonreactors) or *M. bovis* reactors, respectively.

ger than for the BoPPD, the reactivities to both PPDs were robust and diminished at relatively similar rates throughout the 14-year study (19). The close similarities of the BoPPD and *M. avium* subsp. *paratuberculosis* antigen responses would indicate that these mycobacterial species share some dominant antigens that may be capitalized upon by vaccination. The present data suggest that when used as a diagnostic tool for paratuberculosis with the current *M. avium* subsp. *paratuberculosis* antigens, the IFN- γ test would not distinguish vaccinated animals from *M. avium* subsp. *paratuberculosis*-infected animals. In addition, if the IFN- γ test, using BoPPD as the sole antigen, was performed to screen for TB in herds, animals that have been vaccinated for *M. avium* subsp. *paratuberculosis* could be misclassified as TB positive. However, the use of the rEC (ESAT-6/CFP-10) protein complex may provide some discrimination for this diagnostic test, as there was minimal reactivity demonstrated in the IFN- γ test at any time point in the study in vaccinated calves. This suggests that the rEC protein complex may be useful in differentiating *M. avium* subsp. *paratuberculosis* vaccinates from TB-infected animals in the field.

Antibody-based diagnostic tests for Johne's disease are used widely to screen herds, as they have a rapid readout and are cost effective (29). However, vaccination with whole-cell products will induce antibody responses that are very similar to those acquired in natural infection. In the present study, vaccination of calves resulted in the appearance of *M. avium* subsp. *paratuberculosis* antibodies within 3 to 4 months of vaccination, with strong antibody levels sustained throughout the 12-month study. Few studies are available in the literature assessing the temporal effects of vaccination on antibody responses without the added complication of *M. avium* subsp. *paratuberculosis* infection. The induction of *M. avium* subsp. *paratuberculosis* antibody in experimental challenge models has been addressed in a range of ruminant species and demonstrates a pattern of reactivity that is similar to that seen with vaccination (3, 4). With this in mind, the use of whole-cell

vaccines may preclude the use of standard serologic tests to distinguish infected animals from those that have been vaccinated. Vaccination for Johne's disease in the United States must follow guidelines put forth by the USDA and individual states, including the use of an organism-based detection method for screening animals for infection. Developing subunit vaccines would significantly reduce or eliminate some of the troubling aspects of the whole-cell vaccine without sacrificing beneficial properties. A number of putative antigens have been identified from *M. avium* subsp. *paratuberculosis*, and several of these immunogens have been evaluated for use as subunit vaccines, including a 70-kDa heat shock protein, a novel 74F polypeptide, and a mixture of Ag85 and superoxide dismutase proteins. These proteins have demonstrated success in protection against experimental *M. avium* subsp. *paratuberculosis* challenge but have yet to become commercially available (7, 15, 16). Interestingly, a decrease in the total B cell population observed for vaccinated calves after stimulation of cells with *M. avium* subsp. *paratuberculosis* antigens, concomitant with an increase in CD5⁺ and CD45RO⁺ B cells, could signal a shift to a more mature, activated subpopulation responsible for increased antibody production. We did observe that antigen-stimulated CD5 and CD45RO⁺ B cells were maintained throughout the study for vaccinated calves but declined for control calves (data not shown) and that this coincided with the appearance of *M. avium* subsp. *paratuberculosis*-specific antibody.

The bovine tuberculin test, or caudal fold test (CFT), is the most widely used tool for screening herds for bovine TB and is based upon a response to BoPPD. However, the comparative cervical test (CCT) is more definitive, as it characterizes responses to both BoPPD and AvPPD, thereby allowing the discrimination of potential false-positive reactors due to exposure to *M. avium* subsp. *paratuberculosis* and other environmental mycobacteria. In the present study, vaccinated calves demonstrated biased reactivities to AvPPD (18.9 ± 5.9) com-

pared to their reactivities to BoPPD (6.9 ± 0.9). Using the standardized formula for categorization, all vaccinated calves were in the *M. bovis*-negative zone except for one calf that fell into the lower end of the suspect zone for cattle/bison. Without the additional data provided by the AvPPD, it is possible that vaccinated calves would have been misclassified as TB positive, since 4/5 calves had indurations greater than 5 mm to BoPPD. Furthermore, we were able to characterize the status of calves in the present study with serologic diagnostic tools for bovine TB. Vaccinated calves did not react on either the STAT-PAK, DPP VetTB, or DPP BovidTB test, indicating that specific *M. bovis* antigens, such as ESAT-6, CFP-10, and MPB83, could be used to discriminate between bovine TB infections and *M. avium* subsp. *paratuberculosis* vaccines. This is the first application to evaluate these novel TB diagnostic tests in cattle vaccinated against *M. avium* subsp. *paratuberculosis*. Buddle et al. (6) recently reported that deer vaccinated with a heat-killed whole-cell vaccine for *M. avium* subsp. *paratuberculosis* (Sili-rum; Pfizer) demonstrated false-positive results on both the CervidTB STAT-PAK and the DPP VetTB tests at 14 and 26 weeks postvaccination. However, the number of false-positive reactions increased greatly upon the administration of a CCT at 20 weeks, suggesting that the skin test boosted serum antibody levels. Although all sera from vaccinated calves in the present study were negative (below cutoff), one vaccinated calf did develop a weak band on the VetTB STAT-PAK and DPP VetTB tests at 9 and 12 months. Since the CCT was administered to calves at 6 months postvaccination, it is likely that some moderate boosting of antibody response did occur in this animal due to the administration of BoPPD in the skin test.

Host responses to paratuberculosis vaccines are associated with the induction of both Th1 and Th2 immunity as characterized by antigen-specific IFN- γ responses and *M. avium* subsp. *paratuberculosis*-specific serum antibody, respectively (3, 9, 17, 19, 20). In the present study, more extensive evaluation of immunologic parameters was performed to further characterize host immune responses to vaccination but, also, to identify potential markers that might be useful in differentiating vaccinated and infected animals. Robust IFN- γ responses were observed in vaccinated calves, as mentioned above, but secretion of IL-4, IL-10, and IL-12 was not markedly affected by vaccination. A short-term attenuation of antigen-specific IL-4 and IL-10 secretion was observed within the first 3 months after vaccination, but whether this effect is meaningful or not is unknown. Recently, we observed that IL-10 secretion was up-regulated in both naïve and infected calves after *in vitro* stimulation of PBMCs with MPS compared to its secretion with nil stimulation, suggesting that *M. avium* subsp. *paratuberculosis* induces transitory increases in this cytokine that are not dependent upon recall responses to antigen (27).

Largely, T cell populations did not appear to be affected by vaccination, either in fresh (noncultured) PBMCs or in PBMCs cultured with mitogens and antigen preparations. An exception to this was an increase in NK cells in total PBMCs cultured with JPPD late in the study (9 and 12 months). The age of the calves may have had an impact, since increases in NK cells occurred concurrently in both control and vaccinated calves; however, the degree of increase was greater in vaccinated calves. Little work has been done to characterize a role for natural killer cells in *M. avium* subsp. *paratuberculosis* in-

fection, and there has been no documentation of NK cell populations after vaccination. However, it has been demonstrated that NK cells produce IFN- γ in response to *M. bovis* and *M. avium* subsp. *paratuberculosis* antigens in young calves, so perhaps this cell population is helping to maintain the high levels of IFN- γ noted in vaccinated calves and skewing host immune responses to a Th1-mediated response (21).

Vaccination did induce significant changes in activation markers on T cells, with upregulation of both CD25 and CD26 on total T cells and CD4⁺ T cells. CD4 CD25⁺ T cells are highly induced in both experimentally and naturally infected animals, as well as animals vaccinated for paratuberculosis, and represent a highly activated T cell subpopulation (9, 22, 25, 27, 33). Coexpression of CD25 and the transcription factor FoxP3 on CD4⁺ cells defines T regulatory cells, a subpopulation that is critical in controlling inflammatory responses in the host (5). In contrast, little is known about the function of CD26 to define bovine immune cell populations. We previously demonstrated a significant upregulation of CD26 on CD4, CD8, and $\gamma\delta$ T cells within 3 months after experimental infection of calves that was sustained through a 12-month period (27). This activation marker is predominantly expressed by cells with a Th1 phenotype, aligning them with a concerted production of high levels of IFN- γ , as noted in the present study.

Vaccination has become a necessary alternative for the management of paratuberculosis and has been successfully used in control programs worldwide. Concerns about the interpretation of diagnostic tests for the detection of *M. avium* subsp. *paratuberculosis* in vaccinated animals are valid, as the present study demonstrated that vaccination induced sustained IFN- γ and serum antibody responses. It is clear that vaccination with a whole-cell vaccine product induces both Th1- and Th2-mediated immune responses in the host. Markers of host immunity that may distinguish *M. avium* subsp. *paratuberculosis* infection from vaccination remain elusive, but a more thorough understanding of the mixed Th1-Th2 responses may provide a path forward in the identification of a unique marker. In contrast, the novel serologic tests for the detection of serum antibodies and the CCT test may be useful in discriminating between *M. bovis*-infected animals and those vaccinated for paratuberculosis. More extensive evaluation of these diagnostic tools should be undertaken in herds vaccinated for paratuberculosis to fully understand their stringency.

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