Immune responses after oral inoculation of weanling bison or beef calves with a bison or cattle strain of *Mycobacterium avium* subsp. *paratuberculosis*

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SUMMARY

Paratuberculosis is endemic in domestic and wild ruminants worldwide. We designed the following study to compare host immune responses and pathologic changes in beef calves and bison calves after challenge with either a cattle (*Bos taurus*) or bison (*Bison bison*) strain of *Mycobacterium avium* subsp. *paratuberculosis* (*Map*). In the first part of the study, 6 bison and 6 beef calves were orally inoculated over a 2-week period with a cattle isolate of *Map*. In the second part, 6 bison and 6 beef calves were similarly inoculated with a bison strain of *Map*. Throughout each of the studies, blood and fecal samples were taken monthly for a 6-month infection period. Tissue samples were obtained at necropsy for culture and histopathologic analyses. Results from this study demonstrated that bison calves were more susceptible to tissue colonization than beef calves, regardless of bacterial strain. Although lesions were minimal they were most apparent in the jejunum and distal ileum. Interferon-gamma responses were noted in some calves by one month post-inoculation and were sustained longer in beef calves after challenge with the bison isolate. Antibody was not detected in either beef or bison calves during the 6-month infection period. These results indicate that the host response to strains of *Map* may differ between ruminant species.

INTRODUCTION

Paratuberculosis (Johne’s disease), a chronic enteritis caused by the acid-fast bacterium, *Mycobacterium avium* subsp. *paratuberculosis* (*Map*), has been documented in domestic and wild ruminants worldwide. It has been reported in captive ruminants maintained on game farms or in zoos such as red deer, fallow deer, sika deer, white-tailed deer, tule elk, and moose (4-7,9). Paratuberculosis has recently been reported in free-ranging North American bison in the western United States (1,2). Although bison develop signs of unthriftiness and suffer from weight loss, other clinical signs of disease such as hypoproteinemia are lacking (10). In addition, it is difficult to culture *Map* from the feces of infected bison for definitive diagnosis and serum antibody is negligible. This suggests that the host response to *Map* in bison may differ from the host response in cattle. The objectives of this study were to evaluate potential differences in homologous and heterologous host responses to bison and cattle strains of *Map*.

MATERIALS AND METHODS

Animals. Weanling bison and beef calves were obtained from local farms in Iowa. The farms had no reportable incidence of Johne’s disease in their herds within the last 5 years and were assumed to be free of Johne’s disease. Prior to acquisition and shipment to the National Animal Disease Center, calves were screened for *Map* infection using the whole blood IFN-gamma assay. Only calves that had negative responses to both *Map* antigens and *M. avium* PPD were selected for use in the study. Bison or beef calves were each obtained from a single source. Calves were housed 2 animals per pen in a BL-2 containment barn at the NADC. Upon receipt, calves were dehorned, wormed and acclimated to their diet and environment for 2 weeks prior to initiation of the study. Calves were fed a mixed ration containing corn, wheat mids, and soybean meal for the term of the study.

Oral inoculation. Due to limited containment barn space the study was segregated into two phases, each with 6 bison calves and 6 beef calves. In the first phase of the study, calves were inoculated via stomach tube with live *Map* isolated from mucosal scrapings from the ileum of a cow with clinical paratuberculosis. The bacterial isolate was propagated in M7H9 medium with OADC (Becton-Dickinson, Franklin Lakes, NJ) and 2 mg/l of mycobactin J (Allied Monitor, Fayetteville, MO) and harvested in the log phase of growth. The bacteria were
pelleted by centrifugation at 7500 rpm, washed with 0.15 M phosphate-buffered saline (PBS; pH 7.4) two times and then resuspended in sterile PBS to a final concentration of 1 x 10^6 cfu/ml. Calves were inoculated with 60 mls of bacterial preparation on days 1, 2, 3, 7 and 14 of the study. In the second phase of the study, the protocol was the same except the isolate of Map was obtained from a bison cow with clinical paratuberculosis.

**Sampling.** Blood and fecal samples were obtained from calves for 3 consecutive days prior to inoculation with the bacteria (d -3, -2 and -1) and then on days 7, 14, 21, and 28 of the study and every 30 days thereafter throughout the 6-month infection period.

Fecal culture for Map. Fecal samples (2 g) were processed by the NADC centrifugation and double-decontamination method previously described (8).

**Tissue culture and histopathologic analyses for Map.** Sections of duodenum, jejunum, ileum and their associated lymph nodes were obtained at necropsy. Sections of cecum, spiral, transverse and descending colon as well as colic, hepatic, and iliac lymph nodes were also taken. Portions of each tissue were weighed and homogenized in 0.75 % hexadecylpyridinium chloride solution by use of a stomacher for 1 minute and allowed to stand overnight to decontaminate the cultures. Dilutions of individual tissue homogenates were inoculated onto Herrold’s egg yolk medium (HEYM; NADC) containing 2 mg/l of mycobactin J. After 12 weeks of incubation at 37 ºC, viable organisms were determined by counting the number of colonies on the agar slants. Sections of tissue were also fixed by immersion in zinc formalin. Tissues were routinely processed, embedded in paraffin, cut at 4-6 µm, stained with hematoxylin and eosin (HE) and Ziehl-Neelsen (ZN) by conventional methods, and examined by light microscopy.

**PCR analyses on fecal and tissue samples.** Confirmation of colonies on agar slants was performed by PCR analysis. Briefly, agar slants were flooded with 1 ml of sterile 1 mM Tris-0.05 mM EDTA buffer (pH 7.6) and slants were scraped. The solution was then decanted into a sterile microfuge tube and the tubes placed within a boiling water bath for 10 minutes to release the DNA from the bacteria. DNA was then added to a master mix cocktail containing primers to the IS900 gene of Map and run through a thermal cycler. PCR amplicons and a 50-1000 bp marker (BioWhittaker Molecular Applications, Rockland, ME) were then electrophoresed in a 4 % NuSieve 3:1 Plus agarose gel (FMC Bioproducts, Rockland, ME) in 1X Tris-Borate-EDTA (1 M Tris-HCl, 0.9 M Boric acid, 0.01 M EDTA; GibCO BRL) buffer at 65 V for 1 h. Gels were stained with ethidium bromide, visualized and photographed on a Bio-Rad Gel Doc 1000 Imager System (Hercules, CA).

**IFN-gamma analysis.** One ml aliquots of whole blood obtained in sodium heparin tubes from each animal were pipetted into each of 6 wells of 24-well tissue culture plates (Becton-Dickinson, Franklin Lakes, NJ). Blood samples were cultured alone (nonstimulated) or with concanavalin A or pokeweed mitogen (ConA, PWM; Sigma Chemical Co., St. Louis, MO) at a concentration of 10 µg/ml. The nonstimulated culture was included to evaluate background absorbance in each sample. Samples were stimulated with ConA or PWM, T-cell dependent mitogens, as positive controls to provide information about the ability of the cells to respond to stimulation and secrete IFN-γ nonspecifically. Samples were also plated with avium purified protein derivative (AvPPD; Commonwealth Serum Laboratories, Victoria, Australia), bovis purified protein derivative (BoPPD; CSL), a johnin purified protein derivative (JPDP; National Veterinary Services Laboratories, Ames, IA) or a whole cell sonicate of Map (strain 19698, MpS; NADC, Ames, IA). Final concentration of each PPD or MpS in culture was 10 µg/ml. Cultures were incubated for 18 hours at 39 ºC in a 5 % CO₂ humidified atmosphere. Plates were centrifuged at 500 x g for 15 min and plasma was harvested from each well. Plasma samples were frozen at -20 ºC until analyzed for IFN-γ concentration by ELISA using a commercial kit (Bovigam, BIOCOR, Omaha, NE). A sample was determined to be positive if the absorbance of the stimulated sample (either mitogen or antigen) was 0.100 abs units greater than the absorbance achieved for the nonstimulated control well for that animal. This classification of positive reaction was extracted from similar interpretations reported by researchers who have used the IFN-γ assay for detection of tuberculosis in cattle (3,11).

**Lymphocyte proliferation.** PBMC were resuspended in culture medium (RPMI-1640 containing 25 mM HEPES, 2 mM L-glutamine, penicillin G [200 U/ml], and streptomycin sulfate [200 µg/ml]) to a final concentration of 2 x 10^6 cells/ml. Cell viability was determined to be > 95 % by use of propidium iodide exclusion. PBMC were plated at a density of 2 X 10^5 cells/well in 96-well tissue culture plates in culture medium containing 10 % fetal bovine serum (FBS). Media for mitogenic stimulation contained 10 µg of ConA/ml, phytohemagglutinin (PHA)/ml, or PWM/ml. Medium for antigenic stimulation contained 10 µg of MpS/ml. Plates were incubated for 72 (mitogens) or 120 (MpS) hours at 39 ºC in 5 % CO₂, then [³H]methylthymidine
(5 µCi/well) was added for an additional 18 hours. Cells were harvested on filtermats using a Tomtec harvester (Tomtec, Orange, CT) and counted on a Wallac 1450 Microbeta Plus liquid scintillation counter (Wallac, Gaithersburg, MD).

**Map-specific ELISA.** Plates were coated with a whole cell sonicate preparation of Map (cattle isolate) and incubated overnight in a humidified atmosphere at 4 °C. After 3 consecutive washes with PBS containing 0.1 % Tween 80 (Difco Laboratories, Detroit, MI), plates were incubated for 30 min at 39 °C with 1 % gelatin to block nonspecific binding sites. Plates were then washed 3 times with PBS-Tween solution with 3-min soak periods between each washing. Test sera were diluted 1:400 in PBS, added to wells and incubated for 1 h at 39 °C. Plates were washed 3X with PBS-Tween as described and incubated for 1 h at 39 °C with mouse anti-bovine IgG (VMRD). After washing, sheep anti-mouse Ig, biotinylated F(ab')2 fragment (Amersham, Arlington Heights, IL) was added to wells and incubated for 2 h at 39 °C. Plates were washed and streptavidin biotinylated peroxidase complex (Amersham, MR7000 Dynatech plate reader (Dynatech, Chantilly, VA).

**RESULTS**

Results from the culture of Map from tissues of calves are presented in Table 1. After challenge with the cattle isolate, the jejunal region and the associated lymph nodes were most commonly colonized in bison calves (5 of 6 calves) and less frequently in beef calves. In addition, the distal ileum was affected in 50 % of the bison calves and only 17 % of beef calves. The ileocecal LN was affected in 5 of 6 beef calves but only 1 of 6 bison calves after inoculation with the cattle isolate although the ileal LN was negative for both calf groups. Conversely, colonization of the duodenum, jejunum and mid-ileum was more significant in beef calves compared to bison calves after challenge with the bison isolate. The ileal LN and ileocecal LN were colonized to a higher degree in beef calves than in bison calves.

**Table 1.** Recovery of viable Map from tissues of beef or bison calves after 6 months of infection with either a cattle or bison isolate.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Cattle isolate</th>
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<th>Bison isolate</th>
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<tbody>
<tr>
<td></td>
<td>Beef calves</td>
<td>Bison calves</td>
<td>Beef calves</td>
<td>Bison calves</td>
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<tr>
<td>Duodenum/LN</td>
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<td>1/6</td>
<td>5/6</td>
<td>2/5</td>
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<tr>
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<td>4/6</td>
<td>4/6</td>
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<tr>
<td>Mid-Jejunum/LN</td>
<td>1/6</td>
<td>3/6</td>
<td>4/6</td>
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<td>5/6</td>
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<tr>
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<tr>
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<td>Ileocecal LN</td>
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<td>1/6</td>
<td>4/6</td>
<td>0/5</td>
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<td>3/5</td>
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<tr>
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<td>Hepatic LN</td>
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<td>1/6</td>
<td>2/6</td>
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</table>

In general, the burden of infection within a positive tissue was low (< 10 cfu/g). Histopathologic lesions were negligible in the tissues examined in this study, however, bison calves had more consistent evidence of typical granulomatous lesions associated with Map infection.

Lymphocyte proliferative responses to T-cell mitogens, ConA, PWM, and PHAP were significantly (P < 0.05) higher for bison calves in both parts of the study (Figure 1). Beef calves had higher (P < 0.05) antigen-specific responses to JPPD than bison calves throughout the study but response levels were not different than those
observed for baseline proliferation so were unlikely to be biologically relevant.

Disparate effects of calf breed on IFN-\(\gamma\) production were noted with higher (\(P < 0.05\)) ConA responses noted for beef calves and higher (\(P < 0.05\)) PWM responses noted for bison calves in both phases of the study (Figure 2). Overall, antigen-specific IFN-\(\gamma\) production was higher (\(P < 0.05\)) for beef calves compared to bison calves regardless of bacterial isolate used for challenge (Figure 2).

There were no detectable serum antibodies (IgM, IgG) to \textit{Map} for bison or beef calves during either of the 6-month infection periods (data not shown).

**DISCUSSION**

Calves in the present study were only infected for 6 months yet there were clear signs of subclinical infection within both beef and bison calves regardless of inoculum used. Fecal shedding was minimal but this was expected in a short-term experimental infection. Culture of \textit{Map} from the tissues of infected calves was much more informative and delineated clear differences between beef and bison calves in the colonization of tissues. Challenge with heterologous strains of \textit{Map} resulted in major differences in the susceptibility to infection between calf species with predominant colonization occurring in the jejunal tissues and associated lymph nodes. These results suggest that exposure of naive animals to isolates from different species may result in greater virulence and increased pathogenesis. This study demonstrates that cross-species transmission of \textit{Map} in bison is not only possible but also highly likely. In addition, clinical signs of disease in bison may be reduced compared to cattle because of enhanced T-cell function allowing for containment of infection.

**REFERENCES**


