Short communication

Depletion of CD4 T lymphocytes at the time of infection with *M. avium* subsp. *paratuberculosis* does not accelerate disease progression

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**A B S T R A C T**

A calf model was used to determine if the depletion of CD4 T cells prior to inoculation of *Mycobacterium avium* subsp. *paratuberculosis* (*Map*) would delay development of an immune response to *Map* and accelerate disease progression. Ileal cannals were surgically implanted in 5 bull calves at 2 months of age. Two calves were depleted of CD4 T cells by intravenous injection of anti-bovine CD4 antibody administered 24 h prior to inoculation with *Map*. The two CD4-depleted calves and one non-depleted calf were inoculated via ileal cannula with 1 × 10\(^8\) cfu live *Map* every 3 days for a total of 4 inoculations. Two additional calves served as non-depleted and uninfected controls. Injection with the anti-CD4 mAb reduced the frequency of CD4 T cells from a pre-depletion average of 15% to less than 1% in PBMC at 24 h. However, a consistent proliferative response dominated by CD4 T cells, developed in both treated and untreated calves over the course of the 6-month study period. Recovery of *Map* from serial biopsies obtained from the CD4-depleted and non-depleted calves after *Map* infection did not differ. In addition, CD4 depletion did not increase the level of *Map* shed in the feces over the non-depleted animal.

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1. **Introduction**

*Mycobacterium avium* subsp. *paratuberculosis* (*Map*), the causative agent of paratuberculosis (Johne’s disease [JD]), has been isolated from many ruminant and non-ruminant species including humans (Rideout et al., 2003). Johne’s disease is a disease of major concern to dairy industries worldwide. There is also concern that *Map* is zoonotic and may be involved in the pathogenesis of Crohn’s disease, a devastating intestinal disease of humans (Sartor, 2006). Oral exposure of cattle to *Map* leads to the development of a latent infection and an immune response that controls but does not eliminate the pathogen. Well before the appearance of clinical signs, animals begin shedding bacteria in their feces, contaminating the environment and exposing herd-mates and offspring. The lack of an effective vaccine has made it difficult to control the spread of JD (Harris and Barletta, 2001; Nielsen and Toft, 2008; Nielsen, 2008; Diéguez et al., 2009). Progress in developing such a vaccine has been impeded by a lack of understanding of the mechanisms regulating induction, expression, and persistence of protective immunity and also understanding...
how pathogenic mycobacteria persist and dysregulate protective immunity. Efforts to address this problem by developing a model where disease progression can be studied over a shorter period of time have shown the initial immune response to Map is resilient and not readily altered to trigger disease progression. Studies have shown oral and tonsillar crypt exposure lead to uniform infection and the development of a strong CD4 T cell response detectable 3 or more months following exposure (Waters et al., 2003; Koo et al., 2004; Stabel et al., 2009). Bacteria persist at a low level in multiple tissues without inducing easily detectable inflammatory lesions for over a year post-infection (Allen et al., 2009). Repeated exposure to large doses of bacteria over time has not appreciably accelerated development of lesions (Stabel et al., 2003; Waters et al., 2003; Koo et al., 2004; Sweeney et al., 2006; Allen et al., 2009). Only microscopic lesions have been detected during this time frame (Stabel et al., 2009). Efforts to accelerate disease progression by suppressing the immune response by pre-treating calves with corticosteroids (dexamethasone) have also been unsuccessful (Stabel et al., 2009). Similar studies examining the effect of glucocorticoids on cattle latently infected with Map did not identify a significant difference in clinical progression of disease (Wentink et al., 1988).

These findings indicate that additional approaches need to be explored to establish a model to study the early events of infection and pathogenesis of JD. Depletion of CD4 T cells could interrupt signaling and delay development of effector CD4 T cells essential for bringing infection under control. Mouse models have clearly shown the importance of CD4 T cells in controlling infection with Mtb and Mtb-BCG (Cooper and Khader, 2008; Winslow et al., 2008). Studies with Map, though less extensive, have yielded findings that indicate the same mechanisms are involved (Stabel and Ackermann, 2002). One study demonstrated that cattle that progressed toward clinical disease had fewer Map specific CD4 T cells than asymptomatic cohorts (Koets et al., 2002). Studies in cattle have shown transient depletion of CD4 and CD8 T cells affects the response to antigens and pathogens (Howard et al., 1989, 2004; Naessens et al., 1998; Valdez et al., 2000, 2001).

We developed a bovine ileal cannulation model to gain further insight into the mechanisms of pathogenesis of JD (Allen et al., 2009). The model provides continuous access to the target tissue and an opportunity to study the early events of infection and any early pathological changes that occur following infection. As a first step to identify the pivotal events that initiate protective immunity, we examined the effect of transiently depleting CD4 T cells to determine if depletion in combination with direct inoculation of Map into the ileum would lead to extensive colonization of the ileum and a decrease in the time to clinical disease.

2. Materials and methods

2.1. Animals

Four Holstein bull calves (n = 4) were obtained from herds in Minnesota at 1–2 days of age. The farms were status level 4 herds enrolled in the Voluntary Bovine Johne’s Control Program, with no incidence of JD in the last 4–5 years. The calves were housed in a Biosafety Level-2 (BL-2) containment barn at the National Animal Disease Center, Ames, IA (NADC) for the duration of the study. One additional Holstein bull calf (n = 1) was obtained from the Washington State University research dairy. The WSU dairy is a closed herd that has had no diagnosed cases JD for over 35 years. The calf was maintained in a BL-2 isolation facility for the duration of the study.
All protocols and procedures were approved by the National Animal Disease Center and Washington State University Institutional Animal Care and Use Committees.

2.2. Bacteria

A “low passage” strain of Map strain K-10 (obtained from Dr. Vivek Kapur) was used in the study. The bacteria were cultured in Middlebrook 7H9 medium supplemented with OADC (BD, Franklin Lakes, NJ) and 2 mg/ml mycobactin J (Allied Monitor, Fayetteville, MO) as previously described and harvested in the log phase of growth (0.2–0.4 Abs405 nm). The bacteria were processed as previously described and resuspended in PBS to an approximate concentration of 1 × 10^10–10^11 cfu/ml prior to use (Stabel et al., 2009).

2.3. Ileal cannulation surgery, CD4 depletion, and Map inoculation

Ileal cannulation surgeries were performed approximately 14 days prior to Map inoculation as previously described (Allen et al., 2009). Samples of blood, feces, and ileal biopsies were collected for analysis prior to inoculation. On day −1, two calves (48 and 49) were administered 200 mg of anti-CD4 monoclonal antibody (ILA11A, IgG2a, 5.9 mg/ml; 33 ml volume: Washington State University Monoclonal Antibody Center) intravenously.

Twenty-four hours later, three calves (47, 48, and 49) were inoculated with 1 × 10^6 cfu of strain K-10 Map (20 ml volume in PBS) and then every 3 days for a total of 4 inoculations (days 0, 3, 6, and 9). To inoculate, a 20 French Foley catheter was inserted into the cannula, directed orally, and inflated with air to obstruct ingesta flow through the ileum during the inoculation process. The inoculum was injected directly into the occluded ileum through the Foley catheter. The catheter was left in place for 1 h to allow the inoculum to remain in contact with the isolated section of ileum (Allen et al., 2009). Two calves (50 and 132) were used as negative controls and were not inoculated. At termination of the study, tissue samples representing the ileum, jejunum, and duodenum and the associated lymph nodes, as well as spleen, liver, and regions of the large intestine were taken at necropsy for culture and microscopic analysis.

Pre-inoculation biopsies and blood samples were taken 1 week before CD 4 depletion and inoculation of Map. Biopsies and blood samples were taken on days −1, 2, 10, 14 and monthly thereafter for 6 months. Biopsy samples were stored in sterile microtubes for PCR, culture, and histopathology. Samples for PCR analysis were stored at −20 °C until processed. Culture samples were processed immediately, and histopathology samples were fixed in 10% neutral buffered formalin. Whole blood (300 ml) was aseptically collected from the jugular vein. The blood was placed into vacutainer bottles containing 100 ml of acid-citrate-dextrose (ACD) for flow cytometric (FC) analysis as previously described (Koo et al., 2004). Five to ten grams of feces were collected directly from the rectum for fecal culture.

2.4. Endoscopic evaluation

An Olympus CV-60 Gastroscope (Center Valley, PA) with an outer diameter of 12.2 mm and a channel diameter of 3.2 mm was used to visualize and document intestinal mucosal changes and obtain biopsies. Following visual examination a 2.8 mm biopsy instrument was inserted via the endoscope port to retrieve 15–20 mucosal and sub-mucosal biopsies. The biopsies were taken from areas within 10 cm of the cannula both oral and aboral (Allen et al., 2009).

2.5. Culture of Map from feces and tissues

Fecal samples (2 g) were processed by a centrifugation and double-decontamination method previously described (Stabel, 1997). Decontaminated samples (200 µl) were dispensed onto HEYM agar slants in replicates of 4 and incubated for 12 weeks at 39 °C. Culture of tissue samples was performed on HEYM utilizing 2 different methods (Stabel, 1997) (Allen et al., 2009). Stabel’s method was used for necropsy samples while Allen’s method was used for biopsy samples. Sections of duodenum, jejunum, ileum and their associated lymph nodes were obtained at necropsy. Sections of spiral, transverse, and descending colon as well as the spleen, ileocecal valve, hepatic and iliac lymph nodes were also taken. Portions of each tissue were weighed and homogenized in 0.75% hexadecylpyridinium chloride solution (25 volumes) by use of a stomacher for 1 min and allowed to stand overnight at room temperature to decontaminate the cultures. Sediments from individual tissue homogenates (100 µl) were inoculated onto BBL™ Herrold’s egg yolk agar slants (HEYM) with mycobactin J, amphotericin, nalidixic acid, and vancomycin (Becton Dickinson and Co., Sparks, MD), 4 tubes per sample. After 12 weeks of incubation at 39 °C, viable organisms were determined by counting the number of colonies on the agar slants. Culture of the biopsy samples involved 500 mg of tissue cut into 5 pieces with a sterile scalpel blade and placed in a sterile 15 ml centrifuge tube containing 5 ml of NaCl solution (NaOH-N-acetyl-L-cysteine). The samples were kept at room temperature for 20–30 min and vortexed for 15 s twice. 10 ml of phosphate buffered saline (PBS) was then added to the sample and vortexed for 5 s. The samples were then centrifuged at 1500 × g for 20 min. The supernatants were discarded and 5 ml of PBS was added to each pellet and vortexed for 5 s. The samples were centrifuged at 1500 × g for 5 min. The supernatants were discarded and 5 ml of PBS added to each pellet and vortexed for 5 s, then centrifuged at 1500 × g for 5 min. The supernatants were again discarded and the pellet was added to 10 ml of 7H9 liquid broth culture medium supplemented with 6.7% para-JEM® GS with 2 µg/ml mycobactin J, 0.05% Tween-80, and Para JEM AS (antibiotic solution) (TREK Diagnostic Systems, Cleveland, OH) and incubated at 37 °C in a shaking incubator (100 rpm). After the samples were incubated for 1 month, 100 µl of the samples were spread over 7H9 solid culture plates supplemented as described above and checked monthly for 6 months.
2.6. Histopathology

Biopsy specimens and tissue samples obtained at necropsy were fixed in 10% neutral buffered formalin and prepared for histopathological analysis as described (Rosadio et al., 1988). Fite's acid fast stain was used to screen for the presence of Map.

2.7. Polymerase chain reaction

DNA extraction from tissue samples was performed as previously described (Allen et al., 2009). Oligonucleotide primers were derived from the DNA insertion sequence IS900, which is unique to Map. Primers 150C and 921 (IS900/150C: Forward 5’- CGCTAATTGAGAGTGCGATTG-3’ and IS900/921: Reverse 5’-ATCAACTCCAGACGCGGCG-3’) were used to amplify a unique 229 bp fragment of the IS900 gene, as described (Vary et al., 1990; Allen et al., 2009).

2.8. Blood processing for tissue culture and flow cytometry

Blood processing for analysis of immune response to Map antigens was performed as described in Allen et al. (2009). For analysis of the immune response to Map antigens, PBMC were obtained from Buffy coat fractions of blood collected in acid citrate dextrose (ACD) separated by density gradient centrifugation with Accu-Paque (density 1.086)/Accurate Chemical & Scientific Corp., Westbury, NY). Residual erythrocytes were lysed with H2O. Dead cells and debris were removed from cultured cells by density gradient centrifugation before use in flow cytometry (FC).

PBMC were re-suspended in RPMI-1640 medium supplemented with 13% bovine calf serum (BCS), 2-ME, and antibiotics and then distributed in T 75 tissue culture flasks (3 x 10⁶ cells/ml). One flask each was stimulated with Johnin PPD (20 μg/ml, RPMI-1640 complete medium). An additional flask was cultured without stimulation. Following culture for 6 days, cells were collected and subjected to density centrifugation to remove dead cells and then labeled for flow cytometry as described (Waters et al., 2003).

3. Results and discussion

The present study examined whether decreasing CD4 T lymphocyte numbers during the inoculation phase would accelerate intestinal colonization by Map and progression toward clinical disease. Thus far, investigations have shown exposure to single and serial massive doses of Map by oral and parenteral routes establishes an infection, but does not shorten the latent period that precedes appearance of clinical signs of disease (Waters et al., 2003; Koo et al., 2004; Stabel et al., 2009). Use of segmented loop, everted sleeve and surgical intervention models have shown bacteria are rapidly taken up by M cells, macrophages (MΦ), dendritic cells (DC) and enterocytes and subsequently disseminated to lymph nodes as quickly as 1 h PI (Momotani et al., 1988; Sigurðardóttir et al., 2004, 2005; Wu et al., 2007). Initial studies with the cannulation model have shown infection is established in ileal tissue by direct inoculation into the ileum (Allen et al., 2009). Bacteria persist at a low level as detected by PCR of serial biopsies, with no evidence of the development of gross or microscopic lesions during the first year PI. The findings indicate that infection is quickly brought under immune control without any overt Map induced pathology.

Efforts to interrupt signaling by transient immunosuppression with a corticosteroid before exposure demonstrated the interaction is not readily modulated (Stabel et al., 2009). Studies in cattle have shown transient depletion of CD4 T cells can modulate the response to pathogens, suggesting transient depletion of CD4 T cells could also delay the initiation of an immune response and allow establishment of a fulminant infection by Map (Howard et al., 2004). In the present study, flow cytometric analysis of PBMC showed CD4 T cells comprised about 15% (8.29–26.5%) of the lymphocytes at the initiation of the study in all calves before CD4 T cell depletion. Intravenous anti-CD4 treatment reduced the CD4 T lymphocytes in PBMC at Day 1 post-treatment from 8.29% to .54% in calf 48 and from 13.8% to .58% in calf 49. In the non-depleted calves, CD4 T lymphocytes changed from 26.05% to 13.33% in calf 47 and 14.3% to 11.44% in calf 50. By day 14, CD4 T cell proportions had returned to near normal levels in the treated animals (Fig. 1). Depletion of CD4 T cells within intestinal mucosa, lamina propria and mesenteric lymph nodes was not examined in this study. Due to the possible significance of local lymphocyte populations in controlling Mycobacterial infections, it would be recommended on subsequent depletion studies to examine these areas. It would also be recommended to have multiple or serial depletions to decrease the CD4 T cells for longer periods of time.

To confirm establishment and severity of Map infections, we performed PCR and culture on serial biopsies, necropsy tissues, and feces. Map DNA was detected in biopsy tissues throughout the trial time points. Calf 47 (inoculated, non-depleted (ND)) had 4 out of 9 sampling time points positive, 48 (inoculated, CD4-depleted (D)) had 3 out of 9 time points positive, 49 (inoculated, depleted (D)) had 4 out of 9 time points positive, and noninfected control calves, 50 and 132, had no positive biopsy samples. Calf 47 (ND) had 4 positive biopsy cultures starting at 2 months post-inoculation until necropsy. Calf 48 (D)
had 3 positive biopsy cultures starting at 2 months post-inoculation. Calf 49 (D) had 1 positive biopsy culture at 2 months post-inoculation. Calf 47 (ND) had positive fecal cultures in 5 out of 11 sample time points. Calf 48 (D) had 0 out of 11, Calf 49 (D) had 4 out of 11, and negative control calves had no positive fecal cultures for Map.

Various tissues taken at necropsy from all three inoculated calves were culture positive. Tissues that were positive were: spiral colon, transceding colon, duodenal lymph node, mid-ileum, proximal jejunum, hepatic lymph node, proximal jejunal lymph node, ileocecal junction, colic lymph node, ileal lymph node, and cecum. Most of the tissues had low colony forming units (<5 cfu/2 g of tissue). Calf 47 (ND) had 95% of the tissues positive for Map (20 of 21) at necropsy, calf 48 (D) had 76% (16 of 21) tissues positive, and calf 49 (D) had 55% (12 of 22) tissues positive. Calves 50 and 132 had no positive samples at necropsy confirming that they were not exposed to Map during the study (Table 1).

We compared the immune responses for calves by analysis of the expression of up-regulated activation molecules on the CD4 T cell subpopulation cultured with and without antigen (PPD). At 6 months post-infection, activation marker CD25 was up-regulated on CD4 CD45RO+ T cells in experimentally inoculated animals with only mild changes in infected control animals (Fig. 2). There were no differences in type or percentage of activated cells at 6 months post-inoculation between inoculated calves that were treated with anti-CD4 mAb and the non-depleted inoculated calf. CD8 memory T lymphocytes also proliferated in response to antigenic stimulation, but the response was less vigorous (data not shown). NK cells were present but they proliferated in cultures with or without antigen in 6 day cultures (data not shown). It is possible that the mild changes observed in the negative control animals were age related as we have observed in our lab (data unpublished) or as described in CD8+ cells by Hogg et al. (2011).

As reported here, transient depletion of CD4 T cells had no detectable effect on the development of an immune response to Map. The level of infection in CD4-depleted animals, as detected by culture and PCR, did not differ from the level of infection detected in the untreated animal. In summary, we found that the immune response is characterized by a CD4 memory T cell response to PPD between 3 and 6 months post-inoculation. We also found that transient depletion of CD4 T cells did not delay development of the immune response and reduce the latent phase of infection that precedes clinical disease. Further studies with more power and a longer depletion duration are clearly needed make firm conclusions and identify the events that are targeted by Map that ultimately dysregulate protective immunity.

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