Immunologic responses to *Mycobacterium avium* subsp. *paratuberculosis* in neonatal calves after oral or intraperitoneal experimental infection

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**ABSTRACT**

The current study was designed to compare experimental oral and intraperitoneal inoculation on early host immune responses to MAP infection. Blood samples were obtained on d -5 and -4, 7, 14, 21, 28, and monthly thereafter for the 12 month term of the study. Isolation of peripheral blood mononuclear cells (PBMC) was performed, followed by incubation with medium only (nonstimulated), concanavalin A (ConA), a whole cell sonicate of MAP (MpS), and johnin purified protein derivative (JPPD) for 24, 48, 72, or 144 hr for determination of cytokine secretion, lymphocyte proliferation, and flow cytometric analyses. Results demonstrated that oral inoculation of calves significantly increased lymphocyte proliferative responses to MpS at 12 months. Secretion of MPS-stimulated iNOS by PBMC was higher for oral infection groups at both 6 and 12 months post-infection compared to control calves. IP calves had the earliest antigen-specific IFN-γ responses at 7 d post-infection, preceding responses noted for other infection groups that followed between 90 and 120 d. Average IL-10 responses to ConA and MPS were higher at 1 and 6 months and declined significantly by 12 months post-infection. At 1 month, Oral and Oral/M calves had higher MPS-stimulated IL-10 than other treatment groups. By 12 months only the Oral/M calves had higher IL-10 secretion than control calves. Intracellular IFN-γ and IL-10 levels were measured for CD4+ and CD8+, and γδ T cell subpopulations. At 3 months post-infection, there was significantly higher IFN-γ in CD4+ cells stimulated with MPS in the Oral treatment. Intracellular IL-10 was higher in CD4+ and CD8+ T cells in Oral and IP calves compared to the other treatments. Results demonstrated that oral inoculation of calves increased lymphocyte proliferative responses and iNOS secretion by PBMC stimulated with MpS. Antigen-specific IFN-γ responses were apparent for all infected calves by 90 d post-inoculation and remained elevated throughout the study. At 1 month, Oral and Oral/M calves had higher MPS-stimulated IL-10 than other treatment groups but IL-10 secretion declined by 12 months for all calves. Intracellular IFN-γ and IL-10 in T cell subpopulations stimulated with MPS was higher for calves in the Oral infection groups. T cell activation markers such as CD25, CD26, CD5, and CD45RO were upregulated in infected calves compared to noninfected controls. These results demonstrate that exposure and infection to MAP will invoke early immunologic responses characterized by IFN-γ, IL-10, and iNOS secretion.

**INTRODUCTION**

Infection models are useful for studying host responses to infection to provide further aid in the development of diagnostic tools and vaccines. Various animal models for paratuberculosis have been developed and studied, including cattle, sheep, goats, deer and other wildlife, as well as rodents such as mice, rats, and rabbits. The majority of experimental models for ruminants have utilized an oral inoculation of live *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in order to establish infection, thereby mimicking the fecal-oral route of transmission generally observed in the field. Attempts to develop a calf model of infection that will transition from subclinical to clinical infection in a more appropriate period of time (12 months or less) have failed (Stabel et al., 2003; Uzonna et al., 2003; Waters et al., 2003). The majority of these studies have utilized laboratory-adapted strains of MAP in the inocula, while a few studies have demonstrated more effective results with recent clinical isolates of MAP (Stewart et al., 2007). Investigators have not previously investigated the potential usefulness of dexamethasone to invoke host immunosuppression prior to oral inoculation with MAP to determine if this would cause more severe infection. In addition, although
intraperitoneal inoculation is common for the MAP mouse model it has not been attempted in larger species as a means of experimental infection. The current study was designed to compare experimental oral and intraperitoneal inoculation on early host immune responses to MAP infection.

MATERIALS AND METHODS

Treatment groups

Treatment groups consisted of: 1) Control, n = 4; 2) Oral, n = 4; 3) Oral/dexamethasone (Oral/DXM), n = 4; 4) Intraperitoneal (IP), n = 4; and 5) Oral/mucosal (Oral/M), n = 3. The Oral and Oral/DXM groups were fed milk replacer containing 1 x 10^10 live M. avium subsp. paratuberculosis, strain K-10, 2x per d for 14 consecutive days. The Oral/DXM group were treated exactly the same as the oral group except the calves were administered 0.25 mg/kg BW dexamethasone IV for 3 day prior to bacterial challenge and on d 28 and 56 post-challenge. Intraperitoneal inoculation of calves was performed d 0, 7, 14, and 21 of the study. The Oral/M calves were inoculated by feeding milk replacer containing live M. avium subsp. paratuberculosis obtained by scraping the ileal mucosa from a clinically infected cow on d 0, 7, and 14 of the study. Throughout the study calves were monitored for lethargy, refusal to eat or drink, respiratory distress, diarrhea, weight loss.

Assays

Cells were cultured at 1.4 x 10^6/mL in 48-well flat-bottomed plates with either medium alone (nonstimulated, NS), with concanavalin A (ConA; 10 µg/ml), pokeweed mitogen (PWM; 10 µg/ml) or with MAP whole cell sonicate (MPS;10 µg/ml) for 24, 48, 72 or 144 hr (depending upon the assay) at 39°C in 5% CO2 in a humidified atmosphere. Cell supernatants were harvested after 24 and 48 hr and frozen at -20°C until analyzed for cytokine production. Cells were harvested and processed after 72 hr for lymphocyte blastogenesis and after 6 d for flow cytometric analyses. Additional blood samples were collected at each time point into vacutainer tubes and serum was harvested and stored at -20°C for antibody titer.

Bovine IFN-γ and IL-10 production were measured in cell-free supernatants by ELISA after stimulation of PBMC with NS, ConA, and MPS for 24 and 48 hr. Similarly, bovine iNOS was measured using a colorimetric assay. Cells were stained for intracellular IFN-γ and IL-10 within CD4+, CD8+, and IFN-γ T cell subpopulations after 48 hr of incubation. Lymphocyte proliferative responses to NS, ConA, PWM, and MPS were assessed by incorporation of ³H-thymidine by PBMC after stimulation of cells for 72 hr. After 6 days of incubation, PBMC were stained for CD4+, CD8+, γδ T cells, B cells, and activation markers, CD25, CD26, CD5, and CD45RO within those cell populations.

RESULTS

Measures of T cell activation such as lymphocyte proliferation and iNOS secretion were upregulated for PBMC isolated from infected calves after stimulation with MPS. Antigen-specific IFN-γ responses were apparent for all infected calves by 90 days post-inoculation and remained elevated throughout the study (Fig. 1).

IFN-γ Secretion by MPS-Stimulated PBMCs

![Fig. 1. IFN-γ secretion by MPS-stimulated PBMCs](image_url)
Interestingly, IP calves had very robust IFN-γ responses by d 7 post-inoculation that remained steady over the 12-month period. Average IL-10 responses to ConA and MPS were higher at 1 and 6 months and declined significantly by 12 months post-infection, regardless of treatment group (Figure 2). At 1 month, Oral/M calves had significantly higher MPS-stimulated IL-10 compared to other treatment groups. By 12 months, only the Oral/M calves had higher IL-10 secretion than control calves. Intracellular IFN-γ and IL-10 levels were measured for CD4+, CD8+, and γδ T cell subpopulations. At 3 months post-infection, there was significantly higher IFN-γ in CD4+ cells stimulated with MPS in the orally inoculated calves. Early in the study (< 3 months) intracellular IL-10 was higher in CD4+ and CD8+ T cells for Oral/M and IP calves compared to the other treatments. By 12 months, intracellular IL-10 was higher in CD4+, CD8+, and γδ T cells from Oral/M calves after stimulation of cells with MPS.

Fig. 2. IL-10 secretion by MPS-stimulated PBMCs

Experimental infection of calves resulted in the up regulation of activation markers on T cell subpopulations with similar expression patterns noted for CD4+, CD8+, and γδ T cell subsets. Figure 3 represents the increased expression of CD25 within the CD4+ subpopulation after stimulation of PBMC with MPS for 6 days. Infection of calves resulted in significant increases in CD25 expression for CD8+ and γδ T cells as well (data not shown). Similar upregulation of CD26, CD45RO, and CD5 activation markers was also noted on total PBMC populations and T cell subpopulations for infected calves throughout the study (data not shown).

Fig. 3. CD4/CD25 subpopulations in MPS-stimulated PBMCs

DISCUSSION
Experimental infection with MAP, by either oral or intraperitoneal routes, resulted in the upregulation of early cell-mediated immune responses by the host as demonstrated by the
robust antigen-specific IFN-γ responses observed for infected calves. IP calves responded by d 7 post-inoculation but calves in the oral infection groups did not respond consistently until d 90 of the study. The recall response to MAP antigen was observed in infected calves by increased expression of activation markers CD25, CD26, CD45RO, and CD5, regardless of T cell subpopulation. IL-10 secretion was higher in the early months of the study and declined steadily thereafter. This does not fit the paradigm of the Th1-Th2 shift in immunity during MAP infection but calves in this study did not demonstrate signs of advanced infection as antibody titers were negligible (data not shown), fecal shedding was minimal, and no clinical signs were observed.

CONCLUSIONS
Oral inoculation with a laboratory-adapted or clinical strain of MAP evoked strong cell-mediated immune responses in the host. IP inoculation may prove useful in the evaluation of acute immunologic responses to MAP infection as responses were similar to those for oral infection methods in this study, particularly in the first months post-inoculation. Useful immunologic markers of subclinical or early MAP infection for cattle as observed in this study were IFN-γ, iNOS, and T cell activation markers, CD25, CD26, CD45RO, and CD5.

REFERENCES