T cell and macrophage responses during natural and experimental disease caused by Mycobacterium avium subspecies paratuberculosis

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T cell and macrophage responses during natural and experimental disease caused by
Mycobacterium avium subspecies paratuberculosis

by

Bruce Vernon Thomsen

A dissertation submitted to the graduate faculty
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This is to certify that the doctoral dissertation of

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has met the dissertation requirements of Iowa State University.

Signature was redacted for privacy.

Co-major Professor

Signature was redacted for privacy.

Co-major Professor

Signature was redacted for privacy.

For the Major Program
To my wife

DeeDee

And my daughters

Danielle and Ashley
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ABSTRACT

The objective of this dissertation was to examine T cell and macrophage responses during natural and experimental disease caused by *Mycobacterium avium* subspecies *paratuberculosis*. In the initial experiment, euthymic BALB/c and athymic nude BALB/c mice were infected with *M. a. paratuberculosis*. Five months post-inoculation, euthymic mice differed from athymic mice in that (1) their hepatic granulomas were smaller, contained fewer bacteria, and produced more inducible nitric oxide synthase (iNOS), and (2) their hepatic macrophages contained fewer bacteria, a higher percentage of degraded bacteria, and increased numbers of primary lysosomes. The study showed that macrophage activation was markedly decreased in the T-cell deficient athymic mice. In the second study, calves from *M. a. paratuberculosis* infected cows and calves from non-infected cows were inoculated with *M. a. paratuberculosis* or saline. The calves were monitored for evidence of exposure to *M. a. paratuberculosis* for 7 months by IFN-γ assays, skin testing, serology, and fecal culture. Calves from dams subclinically infected with *M. a. paratuberculosis* had significantly higher IFN-γ responses to mycobacterial antigens, prior to inoculation on day 1, and cumulatively throughout the experiment than calves from non-infected dams. All of the calves (2) from infected dams and inoculated with *M. a. paratuberculosis* had positive skin test responses and *M. a. paratuberculosis* isolated from tissues at the end of the experiment. This study showed that maternal infection resulted in an increase in IFN-γ production to mycobacterial antigens by their calves. The final study examined the immunohistochemical and ultrastructural pathology of the ileum in bovine Johne's disease and quantified CD3 lymphocytes, CD4 lymphocytes, CD8 lymphocytes, γδ lymphocytes, CD79 lymphocytes, CD68 cells, iNOS and mycobacterial immunoreactivity. Cows with Johne's disease had increased immunoreactivity in epithelial areas for CD3 and γδ antigen, while in the lamina propria there was increased immunoreactivity for CD68 and mycobacterial antigen as compared to control cows. Inducible nitric oxide synthase immunoreactivity occurred only in the
cows with Johne's disease and was limited to macrophages and multinucleate giant cells located in the submucosa and serosa. Ultrastructural analysis of *M. a. paratuberculosis* infected macrophages revealed a hypertrophied vacuolar transport system and numerous structurally intact bacilli. The majority of bacilli were singly within tightly adherent vacuoles. Less frequently there were clusters of two to fourteen bacilli within large loosely adherent electron lucent vacuoles. Collectively these studies suggest 1) T cells increase macrophage activation in BALB/c mice infected with *M. a. paratuberculosis*. 2) T cell responses to mycobacterial antigens are increased in calves from dams subclinically infected with *M. a. paratuberculosis*. 3) T cell populations and macrophage activation are altered in cows with clinical Johne's disease.
CHAPTER 1. GENERAL INTRODUCTION

Introduction

*Mycobacterium avium* subspecies *paratuberculosis* (*M. a. paratuberculosis*) causes Johnne's disease, which was first described in 1894 by H. A. Johne and L. Frothingham.  

*M. a. paratuberculosis* is a gram positive, acid fast, intracellular bacilli that survives and replicates within macrophages.  

Johne's disease occurs naturally throughout the world in domesticated and wild ruminants, rabbits, foxes and rarely other animals such as primates and asses.  

Experimentally *M. a. paratuberculosis* has been shown to infect mice, hamsters, guinea pigs, chickens, and pigs.  

The ability of *M. a. paratuberculosis* to infect and cause disease in humans is uncertain, but recent evidence has strengthened the notion of an association between *M. a. paratuberculosis* and Crohn's disease.  

In cattle, clinical disease occurs after an incubation period of months to years and is characterized by weight loss and diarrhea.  

Affected cattle are afebrile and have a normal appetite despite progressively worsening diarrhea and malabsorption that eventually lead to death in several weeks to months after the onset of clinical signs.  

At necropsy, affected cattle typically have a severe granulomatous enteritis, lymphadenitis, and lymphangitis with large numbers of acid fast bacilli within macrophages and multinucleated giant cells.  

Approximately 22% of United States dairy herds and 7.9% of beef herds contain cattle with evidence of *M. a. paratuberculosis* infection.  

In the US alone, the negative economic impact of Johne's disease is estimated to be 1.5 billion dollars annually.  

If *M. a. paratuberculosis* is ever definitely linked to Crohn's disease, or the general public perceives that there is a link, one would expect a significant decrease in milk, cheese and beef consumption resulting in even greater economic losses to American agriculture.  

The decrease in milk and cheese consumption would likely occur despite the strong scientific evidence that *M. a. paratuberculosis* is killed during the pasteurization
process. \cite{14,15} Even with the limited evidence linking Crohn's to \textit{M. a. paratuberculosis} today, some consumer groups are strongly arguing that meat from cows infected with \textit{M. a. paratuberculosis} is unfit for human consumption and must be rendered. These economic factors and concerns about possible zoonotic disease are the driving forces behind the emerging state and federal programs to control and eradicate Johne's disease.

Research into the pathogenesis of \textit{M. a. paratuberculosis} infections in natural and experimental disease will provide the scientific basis for the successful elimination of Johne's disease. Using current diagnostic testing methods, it is difficult to detect the majority of animals that are infected with \textit{M. a. paratuberculosis}. \cite{16} To effectively develop new diagnostic tests and vaccines needed to control Johne's disease, there must first be an understanding of the intricacies of \textit{M. a. paratuberculosis} infection in both naturally occurring disease and in the animal models used to study \textit{M. a. paratuberculosis}.

The objective of this dissertation was to examine T cell and macrophage interrelationships during experimental and natural disease caused by \textit{M. a. paratuberculosis}. In chapter 3, the study examined the role of inducible nitric oxide synthase (iNOS) and the ultrastructural pathology of \textit{M. a. paratuberculosis} infection in BALB/c mice. In chapter 4, the effect of maternal infection with \textit{M. a. paratuberculosis} on the offspring was examined and the calves' immune responses to the bacteria were followed using two newly developed diagnostic tests. Finally in chapter 5, iNOS production, lymphocyte populations, and ultrastructural pathology of the ileum in naturally occurring bovine Johne's were examined.

\textbf{Dissertation Organization}

The dissertation is composed of a general introduction, literature review, three separate experimental studies, and a general conclusions chapter. The first experiment, chapter 3, has been published in the \textit{Journal of Comparative Pathology}. The second experiment, chapter 4, has been submitted for publication to the \textit{American Journal of Veterinary Research}. The third study, chapter
5. has been prepared for submission to Veterinary Pathology. L B.V. Thomsen was first author on all three papers. N.F. Cheville (co-major professor) was co-author on all three papers, and M.R. Ackermann (co-major professor) was co-author on the first and third papers.

References


CHAPTER 2. LITERATURE REVIEW

Transmission of *M. a. paratuberculosis*

Johne's disease typically begins in susceptible young animals by the ingestion of feces infected with *M. a. paratuberculosis*. Shedding of *M. a. paratuberculosis* in large numbers from animals with clinical and subclinical disease results in the contamination of the calves' feed and environment. Calves can also be infected while nursing udders contaminated with infective feces through infected milk, or colostrum. *M. a. paratuberculosis* has been cultured from milk samples in 11.6% of cows with subclinical Johne's disease. A similar study of cows with subclinical Johne's disease found that 22% of colostral samples and 8% of milk samples were contaminated with *M. a. paratuberculosis*.

A less frequent route of infection is in-utero transmission of *M. a. paratuberculosis*. A 1958 study estimated that in-utero transmission of *M. a. paratuberculosis* to the fetus occurs 37% of the time in cows with clinical Johne's disease. A later study (1989) found that 26% of fetuses were infected in-utero, and estimated that the true risk of vertical transmission of *M. a. paratuberculosis* from cows with clinical disease was between 11% and 40%. Interestingly, no correlation between gestational age and incidence of infection was found. These initial studies suggest that the risk of vertical transmission is significant and disease control programs frequently recommend eliminating these offspring from the herd. This control measure is expensive to implement, so this recommendation was frequently ignored by producers. In 1992, a study examined the rate of fetal infection in cows that were fecal culture positive for *M. a. paratuberculosis*, but had no clinical signs of Johne's disease. In a herd with Johne's disease, subclinical infections make up the largest population of infected animals, so this study is arguably the most relevant for controlling Johne's disease. Sweeney *et al* found that only 8.6% of fetuses from infected dams had been infected in-utero, and all of those infected calves came from cows that were classified as heavy shedders of *M. a. paratuberculosis* in the feces. Logically, the risk of vertical transmission appears to increase as the
levels of bacteria increase in the dam. None of the studies followed the outcome of vertical
transmission in the calf, or how in-utero infection or in-utero antigen exposure affects the immune
responses of the calf. Younger calves are more susceptible to \textit{M. a. paratuberculosis} than older
animals, and the fetal calf's immune system would not be fully developed; therefore, the likely
infective dose might be quite low in fetuses.\textsuperscript{1}

The pathogenesis for in-utero \textit{M. a. paratuberculosis} infection is unknown, but it may be
similar to that of \textit{Brucella abortus}. In brucellosis, the bacteria first infects trophoblastic epithelial
cells, the bacteria is then released into the amniotic fluid, the contaminated amniotic fluid is ingested
by the calf, and the bacteria is taken up by intestinal M cells.\textsuperscript{8}

Once ingested, \textit{M. a. paratuberculosis} has been shown to cross the intestinal barrier via M
cells.\textsuperscript{9,10} In a ligated ileal loop study of calves, bacilli were found within M cells of the follicular
associated epithelium after 20 hours of incubation.\textsuperscript{9} Most bacilli were seen within subepithelial
macrophages.\textsuperscript{9} A more recent (2001) ileal loop study of goat kids found bacilli was within M cells
within 30 minutes of inoculation, and bacilli were also between M cells in the basolateral pocket.\textsuperscript{10}
Within one hour, bacilli were both free and intracellular within macrophages in the underlying lamina
propria.\textsuperscript{10} It is interesting that the closely related pathogen, \textit{M. avium}, has been shown to invade the
host by primarily entering through enterocytes, and not M cells.\textsuperscript{11} \textit{M. a. paratuberculosis} bacilli were
not detected entering enterocytes in either the calf or goat study previously described.\textsuperscript{9,10}

\textbf{The Immune Response to \textit{M. a. paratuberculosis}}

\textit{M. a. paratuberculosis} invokes a complex immune response from the host that changes
dramatically over time as the infection progresses. This response has been studied intensively in
order to develop an effective diagnostic test for \textit{M. a. paratuberculosis} infection. Early subclinical
infections are correlated with strong cell mediated responses and humoral immune responses
predominate later during clinical disease.\textsuperscript{12} The findings of the second study in this dissertation
which examined experimentally inoculated calves were consistent with these previous observations.\textsuperscript{12}
The clinically normal *M. a. paratuberculosis* inoculated calves had IFN-γ and skin test responses to mycobacterial antigens and no detectable humoral responses to *M. a. paratuberculosis*.

Phagocytosis of the bacilli by macrophages is an early step in directing the acquired immune response. Acquired immune responses are divided into cell mediated responses and humoral responses. The cell mediated responses are associated with Th1 cytokine profiles and humoral responses are associated with Th2 cytokine profiles. Cell mediated immune responses are detected by measuring Th1 cytokines, lymphocyte proliferation, delayed-type hypersensitivity responses and other methods. Humoral immune responses are primarily measured by detecting antibodies. The T helper cell cytokine profiles were originally based on murine CD4 T cells and the cytokines they produced during cell mediated or humoral immune responses.\textsuperscript{14} The murine system categorizes Th1 responses as those producing IFN-γ, IL-2, and TNFβ, and Th2 responses as those in which IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 are produced.\textsuperscript{14} The bovine immune response follows similar patterns, but frequently there is considerable overlap between the two types, and certain cytokines appear to be more important in certain species or types of infections.\textsuperscript{14} Besides CD4 T cells, CD8 T cells, γδ T cells, NK cells, and antigen presenting cells also produce both Th1 and Th2 cytokines.\textsuperscript{14}

The two primary cytokines, in the balance of determining whether a naïve lymphocyte will become a Th1 or Th2 cell, are IL-12 and IL-4 respectively.\textsuperscript{14} In cattle, IL-12 has been proven to follow the murine model and shifts the balance toward a Th1 response as shown by increased IFN-γ production.\textsuperscript{15,16} Evidence of the ability of IL-4 to direct a Th2 response in cattle in vitro has been somewhat less convincing and has been found to have only minor effects on directing cytokine production.\textsuperscript{15,16} Monocytes infected with mycobacteria have been shown to produce IL-12, which is consistent with the theory that a *M. a. paratuberculosis* infected macrophage in the lamina propria would initially direct lymphocytes toward a Th1 response.\textsuperscript{17} Th1 cells in response to IL-12 then begin producing IL-2, TNF-β, and IFN-γ, which direct the host immune response against the mycobacteria.\textsuperscript{12}
An important component in the immune response against mycobacteria is macrophage activation by IFN-γ. IFN-γ increases the macrophage's reactive nitrogen and reactive oxygen bactericidal systems, increases antigen presentation via major histocompatibility molecules, and increases numerous other macrophage functions. However, it has been shown that *M. a. paratuberculosis* can down regulate MHC I and MHC II expression on infected macrophages in the presence of IFN-γ and TNF-α. TNF-α also contributes to macrophage activation, and has been shown to be both detrimental and beneficial to *M. a. paratuberculosis* survival within infected macrophages depending on the levels TNF-α. The scavenger receptor pathway is another IFN-γ independent pathway that increases macrophage activation. Macrophage activation, as demonstrated by increased bactericidal activity, is greatest when both IFN-γ dependent and independent pathways are stimulated.

In Johne's disease, IFN-γ production has been studied to increase the understanding of the immunopathogenesis of Johne's disease and as a diagnostic test for Johne's disease. In vitro studies of bovine monocytes have shown that IFN-γ increases nitric oxide production in *M. a. paratuberculosis* infected cells. The increased macrophage activation observed resulted in minor increases in mycobactericidal activity and significant decreases in intracellular replication of *M. a. paratuberculosis*. IFN-γ production in response to mycobacterial antigens has been used to investigate the immunopathogenesis of naturally occurring Johne's disease. Cows with subclinical *M. a. paratuberculosis* infections have greater IFN-γ production in response to mycobacterial and other antigens from peripheral blood mononuclear cells than do clinically infected or non-infected cattle. CD4 T cells are the major producer of IFN-γ, followed by lesser amounts from CD8 T cells and only minimal amounts from γδ T cells. Subclinical infected cows also produced more TNF than clinically infected cows. When ileal tissues and mesenteric lymph nodes of clinical and subclinically *M. a. paratuberculosis* infected cattle were examined for IFN-γ expression, the results
were similar to those seen in the studies using peripheral blood mononuclear cells. The same study also examined IL-4 gene expression and found no differences between clinically and subclinical M. a. *paratuberculosis* infected cattle. In a similar study, M. a. *paratuberculosis* infected sheep were divided into two groups, sheep with few acid fast bacteria within tuberculoid lesions which were compared to sheep with many acid fast bacteria in lepromatous lesions. The sheep with few bacteria had greater IFN-γ and IL-2 responses to mycobacterial antigens than the sheep with many bacteria. Similar results were obtained when cell mediated immunity was measured by lymphocyte proliferation responses instead of IFN-γ responses.

To summarize both the sheep and cattle studies, it appears that a strong Th1 response, as shown by increased IFN-γ in the sheep with tuberculoid lesions or subclinical bovine disease, is associated with low numbers of bacteria compared to animals with low IFN-γ and multibacillary lesions. A similar relationship between Th1 response and low numbers of bacteria is also seen in *M. tuberculosis* and *M. leprae* infections. The mechanism that causes the Th1 immune response that is present for years during subclinical infection to switch to an apparently much less effective Th2 response is currently unknown. Identification of this mechanism is crucial in the understanding of the pathogenesis and possibly developing therapies to prevent clinical Johne's disease.

Methods detecting cell mediated immune responses have the greatest potential to be the early diagnostic tests that are urgently needed to control Johne's disease. The main tests for cell mediated immune responses include IFN-γ assays, delay-type hypersensitivity responses and antigen specific lymphocyte proliferation assays. The majority of studies have concentrated on IFN-γ production by peripheral blood cells to fill this need. IFN-γ assays have had significant success as a diagnostic tool for *M. bovis*, and are currently approved as official bovine tuberculosis tests in a number of countries. One of the first studies to investigate IFN-γ responses as a diagnostic tool for M. a. *paratuberculosis* was Billman-Jacobe et al. Their assay detected 71.8% to 93.3% of subclinically
affected cattle and 100% of clinically affected cattle with a specificity of 97.6%. More recent studies examining mycobacterial antigen induced IFN-γ responses has shown that, when used in conjunction with an antibody test that detects a different but overlapping population of *M. a. paratuberculosis* infected cattle. IFN-γ tests are an effective method to identify infected cattle.

Experimental disease studies with young calves have shown that both infected and control calves have significant IFN-γ responses to *M. a. paratuberculosis, M. bovis, and M. avium* antigens. Although the specificity of the test in young stock is low when used as a single test, repeated tests over several months did show significant differences in IFN-γ responses between calves inoculated with *M. a. paratuberculosis* and calves given saline. A study of goat kids found that IFN-γ responses were a reliable method to detect exposure at eleven weeks post-inoculation, which also suggests this assay has the capability of detecting very early *M. a. paratuberculosis* infection.

The IFN-γ assay continues to improve, but still has significant limitations such as the need for rapid, temperature stable transportation of the sample to the laboratory. Prolonged transportation and extreme temperatures decrease lymphocyte viability which may than render the sample invalid. Using whole blood as the sample instead of isolated mononuclear cells, proper controls to assure lymphocyte viability, and concurrent skin testing have eliminated many of the difficulties in converting this from a research tool to a diagnostic tool. However, the lack of a highly specific *M. a. paratuberculosis* antigen that doesn’t cross react with other mycobacterial species, especially the ubiquitous *M. avium*, continues to be a major problem.

Intradermal skin testing is an in-vivo method to evaluate antigen specific cell mediated immune responses. Skin testing, and the resulting delayed-type hypersensitivity response, has been an extremely effective diagnostic tool in the fight against mycobacterial diseases for over 100 years. In my ongoing collaborative research, skin testing has shown promising results: in the early detection of experimentally inoculated calves, as an adjunct to increase the efficacy of concurrent IFN-γ testing
in a bovine field study, and finally as a useful diagnostic test in pigmy goats, fallow deer, and sheep using a novel *M. a. paratuberculosis* purified protein derivative (PPD). A sensitive and specific skin test or other cell mediated immunity based assay for sheep is especially needed because current methods of ante mortem diagnosis of *M. a. paratuberculosis* by serology or fecal culture are relatively insensitive until the final stages of the disease.*

Purified protein derivatives are cell free, mycobacterial culture extracts composed of a complex mixture of proteins, lipids, carbohydrates and nucleic acids. Many of the antigens within this mixture are common to the three primary mycobacterial PPD’s: bovine tuberculin from *M. bovis*, avian tuberculin from *M. avium*, and johnin. The numerous common antigens significantly hinder the specificity of these tests. Further complicating matters is the situation with Johnin, the PPD manufactured by the USDA for diagnosis of Johne’s disease, which is actually an extract from a *M. avium* culture. Because of the common antigens between mycobacteria, *M. avium* PPD has been used with limited success to diagnose Johne’s disease. *M. avium* PPD has identified calves inoculated with *M. a. paratuberculosis* as early as 150 days post inoculation. Studies have attempted to solve the problem of common antigens within different PPDs by identifying proteins that are unique to each mycobacterial species for each PPD.

When this complex mixture is injected intradermally into a previously sensitized animal, the resulting swelling at the injection site is due to both antigen specific lymphocytes and non-specific immune responses. Cattle, naturally infected or experimentally sensitized with *M. bovis* and then tested with bovine tuberculin, had vascular and perivascular accumulations of γδ T cells and neutrophils, occurring in the first 24 hours. At 48 and 72 hours, macrophages, CD4 T and CD8 T cells dominated the inflammatory response along with abundant edema and fibrin, which is the actual cause of the dermal swelling. Sheep tested with tuberculin had a similar inflammatory pattern, but delayed-type hypersensitivity responses with other reagents such as brucellin have different histological appearances. When a preliminary study, not included in this dissertation examined
Johnin injection sites from cattle with Johne's disease, the results were similar to those seen for tuberculin, based on H and E sections (unpublished data). When IFN-γ, IL-2, IL-4, IL-10, and TNF-α mRNA were examined within bovine tuberculin skin tests, these cytokines were increased in cattle that were either inoculated with *M. bovis* or vaccinated with BCG compared to saline inoculated controls. The *M. bovis* inoculated animals had greater intradermal edema than the vaccinated cattle. This suggests that simply measuring injection site swelling is a more sensitive method to detect infection than measuring cytokine production at the injection site.

As alluded to earlier, bovine tuberculin tests can have an impact on systemic cytokine levels. Skin testing can also temporarily sensitize both immune and non-immune animals. The effects of sensitization can be diminished by waiting at least 42 days before repeating the skin test. Intradermal testing with *M. bovis* PPD on cattle, previously sensitized with killed *M. bovis*, resulted in significantly greater in vitro IFN-γ responses to *M. bovis* PPD but not *M. avium* PPD. These results suggest that skin testing increases the sensitivity of the IFN-γ assay. The increased IFN-γ levels also make it possible to accurately test samples that are collected up to 24 hours before testing, instead of the previous restriction of a maximum of 12 hours from sample collection to testing.

Delayed-type hypersensitivity responses, like IFN-γ responses, are also weaker in the final stages of clinical Johne's disease. One study of *M. a. paratuberculosis* infected sheep examined IFN-γ responses and delayed-type hypersensitivity responses. Eighty seven percent of sheep with positive IFN-γ responses and 80% of sheep with positive skin test responses had tuberculoid lesions and rare bacteria, consistent with a strong cell mediated immune response. In the same study, 36% of sheep with positive IFN-γ responses and 38% with positive skin test responses had lepromatous lesions and numerous bacteria, suggestive of a poor cell mediated response. Similar false negative delayed-type hypersensitivity responses are seen in some animals with *M. bovis* and *M. leprae* infections.
The final test of cell mediated immunity is the lymphocyte proliferation assay. When used as a diagnostic test for *M. a. paratuberculosis*, the test is relatively insensitive, unspecific, difficult, and time consuming to conduct.26

In second study of this dissertation, all three of these methods were used to evaluate the effects of maternal infection with *M. a. paratuberculosis* on the immune responses of the calf. There are no published reports on the effects of maternal infection with *M. a. paratuberculosis* on the immune responses of the calf. This study found that calves, inoculated with saline or *M. a. paratuberculosis* from dams infected with *M. a. paratuberculosis* had significantly higher IFN-γ responses to mycobacterial antigens, on the first day of life and cumulatively throughout the experiment, than calves from non-infected dams.12 These calves also had greater delayed-type hypersensitivity responses than calves from non-infected dams.13 The study suggests that calves from *M. a. paratuberculosis* infected dams can be identified early in life by their immune responses.12

A somewhat related, long term, large, field study examined cows and their calves which were vaccinated with killed *M. a. paratuberculosis* and examined for evidence of *M. a. paratuberculosis* infection at slaughter.52 Cows that were infected with *M. a. paratuberculosis* had slightly but significantly fewer infected offspring than cows with no evidence of *M. a. paratuberculosis* infection.52 The unanswered question is whether maternal vaccination and or maternal infection caused the decreased susceptibility seen in the offspring. Vaccination of pregnant mothers has been shown to decrease the susceptibility of the offspring by mechanisms other than passive antibody transfer.53 For example, human maternal vaccination with tetanus toxoid results in increased antibody responses of the infant, by an undefined mechanism, when the infant is subsequently vaccinated for tetanus.53

Previous studies have confirmed that the bovine fetus and neonate are capable of antigen specific cell mediated immune responses.54-56 Lymphocytes from bovine fetuses, at gestational ages of 210 to 253 days, had similar proliferation responses to a variety of antigens as did lymphocytes
from adult cattle. Fetuses vaccinated during the second trimester with killed \textit{M. bovis} have been shown to develop antigen specific cell mediated immune responses, as measured by skin testing and lymphocyte proliferation, after parturition. A related study showed that these immune responses were suppressed immediately following parturition, and were markedly stronger when the calves were two to three weeks of age.

The evidence of maternal infection altering antigen specific neonatal immune responses in humans is even more convincing. In these studies, neonatal immune responses were altered even in the absence of vertical transmission of the infectious agent. These papers studied antigen specific cytokine production of newborns using umbilical cord blood as the source of lymphocytes. Neonates from mothers that had positive IFN-$\gamma$ responses to \textit{M. tuberculosis}, were compared to neonates of \textit{M. tuberculosis} negative mothers. Neonates from tuberculosis positive mothers had significantly greater IFN-$\gamma$ responses to mycobacterial PPD than neonates from negative mothers. Similar results were seen when neonates of mothers, that had tested positive for schistosomiasis and filariasis, were compared to neonates whose mothers were negative for these helminthes. The neonatal mycobacterial and helminth induced immune responses were consistently similar to the immune responses of their infected mothers. Similarly, maternal trypanosomiasis has also been shown to alter neonatal antigen specific cytokine responses.

What is the mechanism responsible for altered neonatal immune responses occurring in the absence of in-utero infection? The mechanism of fetal in-utero antigen exposure is unknown, but parasitic antigens are present in the serum of infected mothers and these antigens could pass through the placenta to stimulate the fetal immune system. Collectively, the authors suggest that prenatal antigen exposure does not lead to suppression or anergy to these antigens, and may decrease the susceptibility of the infants to these pathogens. This is in conflict with other earlier studies that suggest in-utero antigen exposure leads to immunotolerance, possibly by anergy of antigen specific lymphocytes.
The effects of prenatal helminth antigen exposure on the child's immune responses are not limited to the helminth antigens that they were exposed to in-utero. As in the previous studies, infants of mothers with filariasis and schistosomiasis were compared to infants whose mothers were free of these diseases. Infants from both groups were vaccinated at birth with BCG and the infants were found to be free of both parasitic diseases at ten to fourteen months of life. At 10 to 14 months of age, peripheral blood mononuclear cells from the infants were tested for IFN-γ production to mycobacterial PPD. Infants from non-parasitized mothers produced 26 times more IFN-γ to PPD than did the infants from parasitized mothers. The authors concluded that prenatal antigen exposure to helminth antigens directed the infants immune system toward Th-2 immune response. This Th-2 bias decreased the efficacy of the BCG vaccination.

If parasites in the cow have similar effects on the calf, this would strongly support more aggressive parasite control programs in the adult herd. Realizing of course, that there are two major differences between human studies and their possible application to cattle, the human studies were on non-intestinal helminthes, and the ruminant placenta is markedly different than the human placenta.

Humoral immune responses to M. a. paratuberculosis and other mycobacteria are relatively ineffective, but will be discussed briefly because of their importance in diagnostic testing. ELISAs are one of the most common methods of detecting Johne's disease because the tests are inexpensive, rapid, and samples are easily obtained and stable. Current commercially available tests are very effective at detecting cows with clinical disease and clinically normal cows shedding large numbers of M. a. paratuberculosis, with sensitivities of 88% and 70% respectively. However, these tests only detect 15% of cows shedding low numbers of M. a. paratuberculosis. Because of these poor results, researchers continue to pursue new antigens and other methods such as preabsorbing sera with M. phlei to increase specificity of these tests. Finding abundant antibody only in the cows shedding large numbers of bacteria is consistent with the accepted theory that the humoral immune response to M. a. paratuberculosis occurs in the late stages of the disease. However, a recent study has shown
that this theory is not true for all types of antibody to *M. a. paratuberculosis*. IgG2 antibody to two heat shock proteins and lipoarabinomannan of *M. a. paratuberculosis* were found to be at higher levels in asymptomatic cows than in cows with clinical Johne's disease. In cattle, IgG2 and IgM appear to be associated with Th1 immune responses, which is the type of immune response seen in early subclinical Johne's disease.

**The Role of Inducible Nitric Oxide Synthase**

Nitric oxide is an important bactericidal mechanism for macrophages, as alluded to in the previous discussion. The production of inducible nitric oxide synthase was a significant component of the murine and adult bovine studies in this dissertation, so it will be reviewed in detail. There are three forms of nitric oxide synthase: endothelial nitric oxide synthase and neuronal nitric oxide synthase which are constitutively expressed, and inducible nitric oxide synthase (iNOS) a calcium independent enzyme. Inducible nitric oxide synthase is produced by macrophages, neutrophils, and a large number of other tissues during inflammatory events. The discussion will be concentrated on the role of iNOS in monocytes and macrophages.

Inducible nitric oxide synthase production by the macrophage is stimulated primarily by IFN-γ, TNF-α, IL-1, and IL-2, cytokines which are typically seen in Th-1 type immune responses. Lipopolysaccharide, lipoarabinomannan from the cell wall of *M. tuberculosis*, bacterial exotoxins, and other microbial products also stimulate the production of iNOS.

In man, cell signaling mechanisms for several of the above cytokines have been well characterized. IFN-γ, TNF-α, and IL-1β bind to surface receptors on the macrophage, expression of the iNOS gene is then up regulated by the transcription factors NF-κB, STAT 1α, and AP-1. Post transcriptional regulation of iNOS also occurs via alternative splicing of mRNA. Down regulation of iNOS expression occurs by a variety of cytokines, other inflammatory mediators, and certain pathogens. TGF-β, TGF-α, EGF, and HGF have all been shown to decrease iNOS production. Corticosteroids inhibit iNOS production by interacting with NFκ-β. There is
also negative feedback induced by high levels of nitric oxide. Leishmania spp. and Candida albicans are two pathogens that have been shown to inhibit iNOS production.

iNOS catalyzes the conversion of L-arginine, oxygen and NADPH to nitric oxide and citrulline. Nitric oxide and other reactive nitrogen intermediates are free radicals which damage and kill bacteria and protozoa by a variety of mechanisms. They cause lipid peroxidation of membranes, strand breaks in DNA, and deaminate DNA. There are two main chemical pathways to damage bacterial proteins. Nitric oxide can have direct effects by nitrosylation of sulfhydryl groups which disrupts thiol containing iron and zinc complexes. More frequently, nitric oxide combines with super oxide to form the potent free radical peroxynitrite, which results in tyrosine nitration.

Recent studies have elucidated two mechanisms that may protect M. a. paratuberculosis against reactive nitrogen intermediates. In broth cultures M. a. paratuberculosis secrete relatively large amounts of superoxide dismutase compared to nonpathogenic mycobacterium. Superoxide dismutase catalyzes the break down of superoxide into hydrogen peroxide. This would prevent the formation of the toxic free radical peroxynitrite, which is formed from nitric oxide combining with superoxide. Another potential bacterial mechanism for protection against peroxynitrite is the enzyme methionine sulfoxide reductase. Oxidation of methionine by peroxynitrite has been recently shown to be an important host defense mechanism for killing bacteria like Escherichia coli. Methionine sulfoxide reductase repairs methionine groups in the bacteria that have been damaged by oxidation. This study showed that when the gene for methionine sulfoxide reductase was removed from M. tuberculosis and placed in E. coli, the oxidized methionine groups damaged by peroxynitrite in the E.coli were repaired, resulting in increased survival of the E. coli.

Immunoregulation is another important role of nitric oxide and other reactive nitrogen intermediates. Nitric oxide can inhibit T cell proliferation and influence production of IL-1, IL-6, IL-10, IFN-γ, TNFα, TGF-β and other cytokines. Whether nitric oxide promotes either a Th1 or a Th2 immune response has yet to be determined. Nitric oxide has also been shown to have both pro-
apoptotic effects in the thymus and anti-apoptotic effects on macrophages. T cells, B cells, and cosinophils. The immunoregulatory role of iNOS should not be underestimated, as shown in iNOS knock out mouse models infected with *M. avium* and *M. leprae* which had granulomas up to ten times larger than control mice.

The role of nitric oxide in bovine *M. a. paratuberculosis* infections has not been fully defined. Infection of bovine monocytes with *M. a. paratuberculosis* did not result in increased production of nitric oxide by monocytes. When both IFN-γ and lipopolysaccharide were added to the infected monocytes, nitric oxide production increased, but there was little increase in killing of *M. a. paratuberculosis*. A later study showed that *M. a. paratuberculosis* infected macrophages did induce low levels of nitric oxide synthesis. Similar levels were produced by macrophages infected with gram positive bacteria, but much greater levels were produced by macrophages infected with gram negative bacteria. Co-stimulation of the infected macrophages with IFN-γ resulted in a marked increase of nitric oxide synthesis. In the final paper of this dissertation, there was no immunohistochemical evidence of increased iNOS within granulomatous lesions in the ileal lamina propria from cows with clinical Johne’s disease. However, several of the granulomatous lesions within the submucosa and serosa did have iNOS immunoreactivity within macrophages and giant cells. In serial sections stained for mycobacterial antigen, there was abundant immunoreactivity within these same areas. This suggests that there are two functional distinct populations of *M. a. paratuberculosis* infected macrophages within ileum. Why are the macrophages within the lamina propria less activated, as evident by no iNOS immunoreactivity, than macrophage in other locations of ileum on the same cow? I would suggest that the lymphocyte subpopulations in the lamina propria are different than those in the deeper tissues. This statement is supported by the fact that there are large numbers of γδ cells in the mucosal epithelium, and it is unlikely that there are high concentrations of γδ cells in the submucosa and serosa. The difference in iNOS production also raises numerous other important questions about local cytokine production, macrophage activation.
and the effects of increased nitric oxide on the bacilli that will be addressed in the future studies section of this thesis.

Previous iNOS immunohistochemical studies of various spontaneous diseases have given incomplete answers to the role of iNOS in inflammatory diseases of cattle. A retrospective study of bronchopneumonia in cattle caused by a variety of pathogens showed that iNOS immunoreactivity was primarily restricted to the margins of necrotic areas.\textsuperscript{75} These pneumonias with iNOS immunoreactivity were caused by \textit{Arcanobacterium pyogenes} and \textit{Mannheimia haemolytica}.\textsuperscript{77} iNOS staining did not colocalize with immunohistochemical markers used to label macrophages, nor did it co-localize with MHC class II immunoreactivity.\textsuperscript{77} The identity of the cells producing the iNOS was not determined.\textsuperscript{77}

Brain lesions caused by \textit{Listeria monocytogenes} in cattle had iNOS immunoreactivity that co-localized with the bacteria in micro abscesses and glial nodules.\textsuperscript{78} As seen in the previous study, there was a separate population of macrophages with immunoreactivity for MHC-II, but not iNOS.\textsuperscript{78} The authors suggested that there were two distinct populations of macrophages, one of which produced iNOS and one which produce MHC-II.\textsuperscript{78}

The understanding of iNOS function in other mycobacterial infections has been greatly assisted by the use of iNOS knockout mice. This has not been done with \textit{M. a. paratuberculosis}, but would be a very interesting study. iNOS knockout mice infected with \textit{M. tuberculosis}, as expected, died more rapidly and had larger bacterial loads than did the control wild type mice.\textsuperscript{79} A second study demonstrated similar results with \textit{M. bovis} BCG infected iNOS knockout mice.\textsuperscript{80} In addition, the knockout mice had larger granulomas with more necrosis, higher levels of IL-12 and TNF, and lower levels of IFN-\textgamma and acid phosphatase.\textsuperscript{80} They suggested that the absence of iNOS lead to a poorly regulated and excessive Th-1 response mediated by higher levels of IL-12.\textsuperscript{80} The low IFN-\textgamma levels and decreased acid phosphatase were likely due to reduced iNOS stimulation of IFN-\textgamma.
production and activation of macrophages. Both studies showed the importance of iNOS in killing bacteria in the *M. tuberculosis* complex.

A study of iNOS knockout mice infected with *M. avium* produced some remarkable results. The iNOS knockout mice had lower levels of bacteria than did the control mice which is in contradiction to the *M. tuberculosis* and *M. bovis* studies. The knockout mice had larger, better organized granulomas, increased survival of CD4 cells and higher levels of IFN-γ than did the control mice. It would appear that the negative effects of nitric oxide immunosuppression outweigh the benefits of nitric oxide induced bactericidal activity in *M. avium* infections. Also that the influence on IFN-γ by nitric oxide is not completely understood as evidenced by the decreased levels of IFN-γ in the *M. bovis* BCG study and the increased IFN-γ in the *M. avium* study. The results from study of iNOS knockout mice infected with *M. leprae* fell some in between the *M. avium* and *M. tuberculosis* studies. The numbers of *M. leprae* within the lesions only were slightly increased compared to control mice, in contrast to the marked increased in bacteria seen in the tuberculosis infected knockout mice and the decreased numbers of bacteria seen in the *M. avium* infected knockout mice. As in the previous studies of other mycobacteria, the granulomas were markedly larger, up to ten times larger, in the iNOS knockout mice. The role of iNOS as observed in studies of iNOS knockout mice can be either beneficial or detrimental depending on the type of mycobacterial infection. Not surprisingly iNOS, in knockout mice, appears to have neither a significant negative nor positive effect on many diseases such as malaria, trypanosomiasis, chlamydiosis and leishmaniasis.

A final consideration that should not be overlooked when reviewing nitric oxide is the species specific regulation and production of nitric oxide. Mice produced abundant amounts of iNOS and iNOS plays a significant role in many diseases, but even in the late 1990's the role and significance of iNOS in human disease was highly questioned. Now the wide scope and importance if iNOS in humans is beginning to be understood. This is because many stimulators of iNOS production are
species specific. The same can be said for the differences in NOS production, or its product nitric oxide, between mice and cattle. IFN-γ induces abundant NOS production from murine macrophages while it only mildly increases NOS production in bovine macrophages. Murine macrophages produce much more NOS in response to TNFα and lipopolysaccharide than bovine macrophages. Even when working only with bovine mononuclear phagocytes there can be marked differences in NOS production. Bovine monocytes cultured less than one day produce little NOS in response to IFN-γ or lipopolysaccharide compared to bovine monocyte derived macrophages. Macrophages were defined as a monocytes cultured for more than three days, had a loss of peroxidase activity, enhanced phagocytic ability, increased sensitivity to lipopolysaccharide, and morphologic characteristics consistent with a macrophage. There are also differences when comparing closely related ruminants. Caprine and ovine macrophages produced less NOS in vitro to lipopolysaccharide and IFN-γ then did similarly stimulated bovine macrophages. In vivo studies of natural listeriosis confirmed the in vitro results and verified that cattle produce more NOS than sheep or goats.

The Role of γδ T cells

The importance of lymphocytes that express the γδ T cell receptors in the bovine immune response to *M. a. paratuberculosis* was highlighted in the third paper of this dissertation which found that cows with clinical Johne’s disease have increased γδ T cells in the ileum. The role of γδ T cells in the ruminant immune response is poorly understood, but the cells appear to play a significant role in mycobacterial infections. In young ruminants up to 75% of peripheral blood mononuclear cells express γδ T cell receptors and in adults 40% of peripheral blood mononuclear cells express γδ T cell receptors. This is in contrast to humans and mice which have only 7% and 2-3% of γδ peripheral blood mononuclear respectively. Ruminants have two main subsets of γδ T cells, the majority of the γδ T cells in peripheral blood cells express WC1 surface antigen and are also CD2-, CD4- and CD8-. A much smaller population of γδ T cells are WC1-, CD2+, CD4+, and CD8+. 
Interestingly, humans and mice do not have lymphocytes that express WC1 antigen. In calves, the ratio of WC1+ to WC1- γδ T cells varies depending on the tissue examined. Intestinal and splenic γδ lymphocytes of calves are primarily WC1+. Within the intestine of calves, γδ T cells are concentrated within the mucosal epithelium with low numbers in the lamina propria.

γδ T cells have numerous functions and their numbers increase in a variety of diseases of cattle, such as paratuberculosis, tuberculosis, cryptosporidiosis and coccidiosis. γδ T cells are similar to αβ T cells in that they can be cytotoxic and produce cytokines such as IL-2, IL-4, IL-10, IFN-γ, and TNF-α. But unlike CD4 and CD8 αβ T cells, γδ T cell responses are not restricted to antigens presented by MHC class I or class II molecules.

* M. a. paratuberculosis infection results in increased numbers of γδ cells in both natural and experimental disease. The third paper of the dissertation studied naturally infected cows with clinical Johne's disease and found there was a significant increase in γδ T cells within ileal epithelial areas of infected cows compared to control cows. Sheep with clinical Johne's disease that had tuberculoid lesions had greater numbers of γδ T cells in the ileal villi than did control animals. Sheep with lepromatous lesions had a significantly higher percentage of γδ T cells, but differences in absolute numbers of γδ T cells were not significant. When the ratio of CD4 plus CD8 to γδ T cells was examined, both the sheep with lepromatous and tuberculoid lesions had significantly lower ratios in the ileal villi than did the control sheep. Similarly, lambs inoculated with *M. a. paratuberculosis* had greater numbers of γδ T cells in ileal tissue 4 weeks after inoculation. γδ T cells were also increased in mesenteric lymph nodes compared to controls, but the increase was not statistically significant.

Studies of γδ T cells in *M. a. paratuberculosis* infections appear to show an immunoregulatory function for γδ T cells. Both γδ and αβ peripheral blood lymphocytes proliferate in response to *M. a. paratuberculosis* antigen. Proliferation of bovine peripheral blood CD4 T cells...
to *M. a. paratuberculosis* antigen was inhibited when γδ T cells were present, compared to CD4 proliferation in the absence of γδ T cells. A follow-up study by the same authors showed that the γδ T cell inhibition of *M. a. paratuberculosis* CD4 T cell proliferation could be blocked by co-culturing the cells with CD8 T cells. The addition of CD8 T cells also inhibited *M. a. paratuberculosis* induced proliferation of γδ T cells. From these studies it appears the γδ T cells suppress CD4 T cells, and CD8 T cells inhibit γδ T cells. Cell to cell regulation likely is mediated via cytokines such as IFN-γ. *M. a. paratuberculosis* induced large IFN-γ responses from bovine peripheral blood CD4 T cells. small amounts from CD8 T cells, and only minimal amounts from γδ T cells. So it is unlikely that the effects of γδ T cells in Johne’s disease are mediated by IFN-γ.

In bovine *M. bovis* infections, both γδ and αβ T cells have been shown to have marked proliferation responses to *M. bovis* antigens. γδ T cells were also more activated, as measured by increased interleukin 2 receptor, than αβ T cells in cattle with tuberculosis. As in *M. a. paratuberculosis* infections, removing γδ T cells from the mixture of peripheral blood mononuclear cells during *M. bovis* stimulated proliferation assays, resulted in increased proliferation of αβ T cells. This further strengthens the evidence that γδ T cells have a suppressive effect on αβ T cells in bovine mycobacterial infections.

Murine models of Johne's disease also support the theory that γδ T cells may have a negative effect on the host's response to *M. a. paratuberculosis* infection. Mice that are genetically more susceptible to *M. a. paratuberculosis*, such as C57BL/6 mice, have γδ to CD4 T cell ratios than do the more resistant C3H mice. *M. a. paratuberculosis* infection results in increased γδ and CD8 T cells in both strains of mice, however, the γδ to CD4 and CD8 to CD4 T cell ratios continue to remain higher in the more susceptible C57 black mice.
When γδ T cell knockout BALB/c mice were inoculated with *M. a. paratuberculosis*, γδ T cells again showed a negative effect on the immune response to *M. a. paratuberculosis*.

The γδ T cell knockout mice had fewer and smaller hepatic granulomas than the wild type mice. The knockout mice also had lower numbers of bacteria than the control mice. The granulomas in the knockout were considered to be immature because they had fewer epitheloid macrophages than the control mice, and γδ T cells appeared to play a role in the recruitment of other cells such as macrophages into the forming granulomas. Unfortunately for the host, these newly recruited macrophages provide the environment *M. a. paratuberculosis* needs to replicate and survive.

When γδ T cell knockout mice were infected with *M. avium*, granulomas were again smaller in the knockout mice than control mice. The granulomas of γδ T cell knockout mice had fewer neutrophils and less tissue necrosis than did controls. Interestingly, there was no difference in bacterial loads between γδ T cell knockout mice and control mice at the end of the experiment (125 days).

In contrast, *M. bovis* infected fetal bovine-severe combined immunodeficient xenochimeric mice, deleting γδ T cells that express WC-1, had greater necrosis and neutrophils in granulomas than control mice. No differences were seen in bacterial loads between γδ T cell deleted mice and control mice.

It appears that the effect of deleting γδ T cells in mice can either be positive or negative depending on the pathogen. γδ T cell knockout mice were markedly more resistant to *Salmonella choleraesuis* septicemia and death than control mice. While in *Candida albicans* infections, deleting γδ T cells increased the susceptibility and severity of infection in BALB/c mice. Deleting γδ T cells also decreased iNOS production within mucosal lesions. Similarly, γδ T cells deleted mice infected with *Listeria monocytogenes* had a higher fatality rate than control mice. These same authors have also shown that γδ T cells are needed to inhibit the host immune response after the
infection is eliminated to prevent chronic disease.\textsuperscript{106} One proposed mechanism for this result is that activated $\gamma\delta$ T cells kill stimulated macrophages.\textsuperscript{107}

In conclusion, it appears that $\gamma\delta$ T cells can be either beneficial or detrimental to the host immune response depending on the pathogen. During \textit{M. a. paratuberculosis} infections, $\gamma\delta$ T cells are increased during natural disease and appear to be more detrimental than beneficial to the hosts overall immune response possibly by suppression of CD4 T cells or other mechanisms.

Besides an immunoregulatory role, $\gamma\delta$ T cells are also important in maintaining mucosal epithelial integrity.\textsuperscript{108} Activated $\gamma\delta$ T cells appear to aid in the repair of the intestinal epithelium by producing keratinocyte growth factor which increases epithelial cell proliferation.\textsuperscript{108} In a study of dextran sulfate sodium induced colitis in mice, $\gamma\delta$ T cells were concentrated around areas of eroded mucosa and within underlying proliferating crypts showing that they are in the proper location to be stimulating the epithelial cells.\textsuperscript{108} When $\gamma\delta$ T cell knockout mice were given dextran sulfate, colonic lesion resolved more slowly than did the lesions in control mice that did have $\gamma\delta$ T cells.\textsuperscript{108} This further supports the role of $\gamma\delta$ T cells in intestinal epithelial repair.\textsuperscript{108}

\textbf{Ultrastructural Pathology of Johne's Disease}

Electron microscopy proved to be a valuable technique to further the understanding of \textit{M. a. paratuberculosis} infections in vivo in the murine and adult bovine studies of this dissertation. There have been limited ultrastructural studies of bovine Johne's disease.\textsuperscript{109,110} Most studies have concentrated on the infected macrophages in the lamina propria, but subtle changes in other areas have been recorded. Kim \textit{et al} observed the absence of epithelial cells on villi, and crypt epithelial cells enlarged by swollen endoplasmic reticulum and mitochondria. There was also intercellular edema, degenerate epithelial cells, necrotic epithelial cells, and cellular debris within the mucosa.\textsuperscript{110}

Macrophages containing bacteria are typically enlarged by variably sized vesicular structures and may have characteristics of acute cell injury.\textsuperscript{109,110} The vesicular structures were believed to
originating from the host cell. Structurally intact bacteria were found within macrophages, (primarily individually, but also in clusters with phagosomes and phagolysosomes) multinucleate giant cells, neutrophils and rarely extracellular. Bacteria within macrophages typically were surrounded by an electron transparent zone, but interestingly no electron transparent zone was seen around bacteria within neutrophils. Because the electron transparent zone is a buildup of material excreted from the bacteria, it suggests that in neutrophils the bacteria is either degraded or the neutrophil dies and releases the bacteria before the electron transparent zone can form.

Bendixen et al concurrently examined ileum from cows with Johne's disease as described above and bovine M. a. paratuberculosis infected monocytes. Infected macrophages contained numerous cytoplasmic membranous structures similar to those seen in the vivo studies. Within phagosomes, both intact and degraded bacteria were identified; the degraded bacteria were thought to have been from phagocytosis of dead bacteria within the inoculum. There was no apparent increase in degraded bacteria within macrophages at the end of the four week experiment. Bacteria were thought to be free in the perinuclear cytoplasm two days after inoculation.

Ultrastructural findings in natural Johne's disease in goats was similar to the bovine ileal studies, and the numerous vesicular structures within infected macrophages were believed to be dilated golgi and endoplasmic reticulum. Numerous uninfected epitheliod macrophages were described as being enlarged by a large central nucleus, numerous free ribosomes, and prominent mitochondria. Ultrastructural characteristics or macrophages and epitheliod macrophages from the mesenteric lymph nodes were similar to those of the ileum. No other ultrastructural studies have examined the lymph nodes of M. a. paratuberculosis infected animals.

Ultrastructural findings of Johne's disease in a single naturally infected adult sheep were similar to that of the other ruminants previously described. The study is the only study of naturally occurring Johne's disease to use immunogold labeling to detect mycobacterial antigen prior to my current study. The labeling identified both intact bacteria and heterogeneous material in
phagolysosomes. The authors identified two subtypes of infected macrophages: 1) macrophages with large numbers of bacteria that had few lysosomes and little phagolysosomal fusion, and 2) smaller macrophages with fewer bacteria, more lysosomes, and more phagolysosomal fusion.

Murine ultrastructural studies of *M. a. paratuberculosis* are limited. A 1969 study by Smith that concentrated on bacteria morphology confirmed that the bacteria resided in a host cell membrane vacuole. Subsequently *M. a. paratuberculosis* infected BALB/c mice were compared to the more resistant strain C3H. Within the BALB/c mice, the bacteria resided in small phagolysosomes with little acid phosphatase activity, few bacteria, and little lysosomal material compared to the C3H mice. The large C3H phagolysosomes also had larger numbers of degraded bacteria and fewer intact bacteria than the BALB/c phagolysosome. The authors concluded that macrophage activation was greater in the C3H mice.

In all species, an electron transparent zone has been observed surrounding the bacilli. The electron transparent zone is a multilamellar capsular like structure between the bacterial cell wall and the inner surface of the phagosome membrane. The electron transparent zone is loosely attached to the bacterial cell wall and is composed of a poorly defined mixture of polysaccharides and proteins. This material is secreted by the bacteria, builds up within the phagosome forming the electron transparent zone, and may function as a protective mechanism for the bacteria. Both pathogenic and nonpathogenic mycobacterium produce an electron transparent zone, but the layer is thicker in pathogenic bacteria. The development of the electron transparent zone has been studied using *M. avium*. Bone marrow macrophages were inoculated with live *M. avium*, killed *M. avium* or *M. smegmatis*, and the macrophages inoculated with live *M. avium* had the greatest percentage of bacilli with electron transparent zones. Over several time points, the percentage of bacilli with electron transparent zones remained the same for the live *M. avium*, while the percentage of electron transparent zones for the killed *M. avium* and *M. smegmatis* decreased over
time. Whether this means the electron transparent zone is just a by-product of successful survival within a macrophage or is a required defense mechanism that allows the bacteria to survive is unclear.

A reoccurring theme in all of the studies is that when the bacteria are in individual vacuoles, there is relatively little evidence of lysosomal fusion and the bacteria are structurally intact. This observation is supported by an ultrastructural study of M. avium in which coalescence of vacuoles containing a single bacilli into vacuoles with multiple bacteria occurs after activation of the macrophage by IFN-γ and lipopolysaccharide. The more resistant murine strains, such as C3H, readily form large phagolyosomes and rapidly degrade the bacteria. At the opposite end of the spectrum are cattle, where bacteria are primarily in small, individual vacuoles with no evidence of lysosomal fusion. When the bovine macrophages do contain large vesicles with multiple bacteria, frequently there is little evidence of lysosomal fusion or bacterial degradation.

A recent in vitro study of murine J774 macrophages infected with M. a. paratuberculosis confirms that the bacteria can inhibit lysosomal fusion. Phagosomes containing M. a. paratuberculosis co-localized with calcein, a marker of lysosomes, at a significantly lower rate than vacuoles containing nonpathogenic mycobacteria. M. a. paratuberculosis vacuoles also contained lower levels of Lamp 2, a protein found in late endosomes and lysosomes, than vacuole containing nonpathogenic mycobacterium or killed M. a. paratuberculosis. And finally, phagosomes with live M. a. paratuberculosis did not acidify (pH 6.3) as readily as phagosomes containing nonpathogenic bacteria or killed M. a. paratuberculosis (pH 5.2).

One could speculate that the above differences between phagosomes containing live M. a. paratuberculosis versus phagosomes killed M. a. paratuberculosis or nonpathogenic mycobacterium would be greater in an in vitro experiment using bovine macrophages. This is based on the ultrastructural characteristics observed in the BALB/c mice (the source of J774 macrophages) from the first paper in this dissertation compared to the ultrastructural changes observed in cows with clinical Johne’s disease. In the first and third studies of this dissertation BALB/c mouse
macrophages appeared to be more effective at killing *M. a. paratuberculosis* bacilli than cattle as evidenced by fewer bacteria per macrophage, more degraded bacteria, greater numbers of lysosomes, and greater evidence of phagolysosomal fusion. It is acknowledged that these observations do not take into account several factors: 1) the cattle were naturally infected with fields strains while the mice were experimentally infected with ATCC strain 19698. 2) the bovine macrophages were from the ileum while murine macrophages were in the liver. 3) the cattle were in the late clinical phases of Johne's, while the mice had no clinical signs.

There are three additional subtle ultrastructural changes observed in the in vivo murine models compared to the published reports of bovine Johne's disease and the third paper of this dissertation. The murine studies report significant numbers of primary and secondary lysosomes while the bovine reports do not consistently demonstrate large numbers of these structures.

All of the cattle reports describe numerous, small and large, clear to weakly electron dense, multivesicular structures and vacuoles that expand the cytoplasm of the macrophage, a characteristic not seen in mice. In cattle, intact bacteria are often very loosely arranged in these weakly electron dense multivesicular like structures. These facts are a reminder that although murine models are very useful, they do not replicate natural Johne's bovine disease in many ways.

Because *M. a. paratuberculosis* resides within multiple, ultrastructurally distinct types of vacuoles, the term bacteriophorous vacuole may be the most appropriate term for these structures. In the third paper of this dissertation, the term bacteriophorous vacuole was used for vacuoles that contained a structurally intact bacteria that did not have the typical appearance of a phagolysosome. In vitro studies previously discussed have shown strong evidence that *M. a. paratuberculosis* and other mycobacteria modify the development and maturation of the vacuole so that the bacteria can both survive and replicate. This is in distinction to endosomes and phagolysosomes which are degradative organelles and contain evidence of disintegration of the bacterial cell. The term is
analogous to a parasitophorous vacuole, which is used primarily for intracellular protozoa, but has also been used to describe intracellular bacteria.\textsuperscript{125,126}

References


42. Steadham EM, Martin BM, Thoen CO. Production of a *Mycobacterium avium* ssp. *paratuberculosis* purified protein derivative (PPD) and evaluation of potency in guinea pigs. *Biologicals* 2001;Submitted.


100. Veazey RS. Horohov DW. Krahenbuhl JL. et al. Differences in the kinetics of T cell accumulations in C3H/HeN (Bcg-resistant) and C57BL/6 (Bcg-susceptible) mice infected with *Mycobacterium paratuberculosis*. *Comp Immunol Microbiol Infect Dis* 1996:19:289-304.


**CHAPTER 3: T CELL-DEPENDENT INDUCIBLE NITRIC OXIDE SYNTHASE PRODUCTION AND ULTRASTRUCTURAL MORPHOLOGY IN BALB/c MICE INFECTED WITH MYCOBACTERIUM AVIUM SUBSPECIES PARATUBERCULOSIS**

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**Summary**

Euthymic BALB/c and athymic nude BALB/c mice aged 3-8 days were infected intraperitoneally with *Mycobacterium avium* subspecies *paratuberculosis*. ATCC strain 19698. After euthanasia at 5 months post-inoculation, hepatic granulomas were evaluated by morphometric analysis of digital images captured from light microscopy sections, by electron microscopy and by immunohistochemical methods. Euthymic mice differed from athymic mice in that (1) their hepatic granulomas were smaller, contained fewer bacteria, and produced more inducible nitric oxide synthase, and (2) their hepatic macrophages contained fewer bacteria, a higher percentage of degraded bacteria, and increased numbers of primary lysosomes. The study showed that macrophage activation was markedly less in the T-cell deficient athymic mice than in the euthymic mice.

**Introduction**

*Mycobacterium avium* subspecies *paratuberculosis* (*M. a. paratuberculosis*) causes Johne's disease, a chronic granulomatous enteritis of cattle, sheep and other ruminants. Clinically infected animals develop chronic diarrhoea or weight loss, or both, months to years after initial exposure. The disease is endemic in much of the world and causes significant economic loss (Clarke, 1997). Some authors have also suggested that *M. a. paratuberculosis* plays a role in the aetiology of Crohn's disease in man (Hermon-Taylor et al., 2000).

*M. a. paratuberculosis* is an intracellular bacterium that infects macrophages within the intestine, lymph nodes and liver (Clarke, 1997). After ingestion by macrophages, the bacteria are located intracellularly within phagosomes. They survive and replicate within the phagosomes, in spite
of the potent bactericidal properties of macrophages (associated with acidification, reactive nitrogen intermediates, reactive oxygen intermediates, and lysosomal enzymes) (Clarke, 1997).

Primary lysosomes are cytoplasmic electron-dense vacuoles that contain acid phosphatase and other enzymes. Primary lysosomes fuse with phagosomes, the enzymes being used to degrade the phagosomal contents. The combined structure of a primary lysosome and phagosome is a secondary lysosome. Such structures are also termed phagolysosomes when the vacuolar contents are known to consist of exogenous material (bacteria) rather than endogenous autophagosomal material (Cheville, 1994).

Inducible nitric oxide synthase is used by the macrophage to produce nitric oxide and other potent bactericidal reactive nitrogen intermediates (Bogdan et al., 2000). Reactive nitrogen intermediates are free radicals which can deaminate bacterial DNA, peroxidize bacterial lipids, and alter bacterial proteins by nitrosylation of thiol groups or nitration of tyrosine residues (Fang, 1997). Nitric oxide also has multiple immunoregulatory effects, such as inhibiting T-cell proliferation (Bingisser et al., 1998) and inhibiting interferon (IFN)-γ production (Taylor-Robinson et al., 1994).

The prolonged incubation period of Johne's disease in natural hosts has resulted in the development of a number of animal models, including those based on the use of various strains of mice (Frelier et al., 1990). BALB/c mice which are inherently susceptible to M. a. paratuberculosis (Hamilton et al., 1989; Chiodini and Buergelt, 1993), are classified as BCG (Bacille Calmette-Guérin)-sensitive because of their lack of natural resistance-associated protein 1, a lack that makes them ideal models for intracellular bacterial infections (Gros et al., 1981; Malo et al., 1994). In the athymic nude strain of BALB/c mice, the immune system is further weakened by a T-cell deficiency. Both euthymic and athymic BALB/c mice, however, develop intestinal lesions similar to ruminants after infection with M. a. paratuberculosis.

In the study reported here, euthymic and athymic BALB/c mice infected with M. a. paratuberculosis were used to investigate the effect of T-cell deficiency on (1) inducible nitric oxide
synthase (iNOS) production (2) the numbers of primary and secondary lysosomes within infected macrophages. and (3) the numbers of intact and degraded bacteria within infected macrophages.

Materials and Methods

Experimental Design

The care and use of the mice was approved by the Iowa State University Committee on Animal Care. Eight euthymic BALB/c (BALB/cAnCrlBR) and 10 athymic nude (BALB/cAnNCrl-nuBR) infant mice, with their dams (Charles River Laboratories, Wilmington, Massachusetts, USA), were housed in a specific pathogen-free facility at Iowa State University. At 3 to 7 days of age. the infant mice were given a single intraperitoneal injection of approximately \(3 \times 10^7\) colony-forming units of \(M. a. \) paratuberculosis (ATCC strain 19698), suspended in 0.1 ml saline. Additionally, 10 euthymic and 6 athymic infant mice, used as controls, were given 0.1 ml saline intraperitoneally. Five months after inoculation the mice were killed and subjected to necropsy. All mice were clinically normal and had normal faeces for the duration of the experiment.

Histopathology. Immunohistochemistry and Image Analysis

Samples of liver, spleen, pancreas, duodenum, jejunum, ileum, caecum and colon were fixed in 10% neutral buffered formalin, processed by routine methods, embedded in paraffin wax and sectioned. Sections (5 μm) were stained with haematoxylin and eosin (HE) and Ziehl-Neelsen (acid-fast). For iNOS immunohistochemistry, sections (3 μm) were labelled with a rabbit polyclonal antiserum (Upstate Biotechnology, Lake Placid, New York, USA) specific for the partial fusion protein derived from the N-terminus of murine iNOS. Briefly, the sections were dewaxed, rehydrated, and then blocked with 10% normal goat serum and 3% bovine serum albumin. The primary antibody was diluted 1 in 10 000 and incubated with the sections for 72 h at 4 C. The sections were blocked with 2% hydrogen peroxide for 40 min followed by treatment with 1 in 200 biotinylated goat anti-rabbit IgG secondary antibody (KPL, Gaithersburg, Maryland, USA) for 45 min. Peroxidase-conjugated streptavidin (BioGenex, San Ramon, California, USA) was applied for 45 min, followed
by NovaRED substrate (Vector Labs. Burlingame California. USA) for 5 min. The slides were counterstained with Harris's haematoxylin. In negative controls, normal rabbit IgG replaced the primary antibody.

Five random fields of liver sections were electronically photographed (Zeiss axiophot microscope with the x10 objective and x1.25 optivar) combined with a Sony DXC-3000A colour video camera (Itasca, Illinois, USA), a Scion CG-7 frame grabber board (Frederick, Maryland, USA) and an Apple PowerMac G3 (Cupertino, California, USA). The images were edited and analysed with Adobe Photoshop (San Jose, California, USA) and Scanalytics IPLab software (Fairfax, Virginia, USA). The area of inflammatory foci within the hepatic parenchyma was measured and compared with the total area of the field of view. A mean of the total area affected was calculated from the five random images for each mouse.

Image analysis measuring iNOS labelling within hepatic granulomas was performed as described above, except that a x40 objective was used. The total area of positive iNOS labelling was compared with the total area of the granuloma. The same slides were then stained by Ziehl-Neelsen and the same five granulomas were photographed again. Area of acid-fast staining was compared with the area of the granuloma. The mean percentages of iNOS labelling area and bacterial staining area per granuloma were then calculated.

Duodenal lesions were evaluated subjectively and lesions were scored, after examining three x10 fields of the HE-stained sections, according to the following scale: 1. duodenal sections with no lesions; 2. duodenal sections with a slight increase in macrophages, lymphocytes or neutrophils, but no significant widening of the lamina propria; 3. duodenal sections in which 1-10% of villi had a two-fold or greater increase in width of the lamina propria due to increased inflammatory cells; 4. duodenal sections in which 11-50% of villi had a two-fold or greater increase in width of the lamina propria; and 5. those in which >50% of villi were widened at least two-fold.

Electron Microscopy
Samples of liver were fixed in 2.5 % glutaraldehyde for 4 h and then transferred to 0.1 M cacodylate buffer. Tissues were post-fixed in 1% osmium tetroxide, dehydrated and embedded in Eponate 12 resin (Ted Pella, Inc., Redding, California, USA). Sections (1 µm) were stained with toluidine blue, examined, and ultrathin sections were made of selected areas. These sections were stained with uranyl acetate and lead citrate, coated with carbon, and examined with a Philips 410 transmission electron microscope (TEM) (FEI Co., Hillsboro, Oregon, USA). One mouse per cage was randomly selected, and the first four hepatic macrophages that contained bacteria, defined cell margins, and nuclei were photographed at x6 900 (print magnification x17 940). Numbers of intact bacteria, degraded bacteria, primary lysosomes and secondary lysosomes were determined by visual counts of micrographs. The following definitions and morphological characteristics were used to define cell structures. *Intact bacteria*: rod-shaped, approximately 0.3-0.6 x 1-4 µm, with an intact electron-dense cell wall; an internal structure composed of electron-dense cytoplasmic granules occupying most of the cytoplasm, lipid bodies and nuclei: size, shape and internal structure consistent with the genus *Mycobacterium*. *Degraded bacteria*: fragmented or possessing an irregular cell wall. *Primary lysosome*: a spherical, membrane bounded, cytoplasmic vesicle (100-300 nm) with homogeneous, finely granular, electron-dense contents. *Secondary lysosome*: an irregularly shaped, membrane bounded, cytoplasmic vesicle (300 to 2300 nm) that contained degraded or non-degraded bacteria or other heterogeneous material and homogeneous, finely granular, electron-dense material. *Statistics* Means, standard error of means, and sources of variation were determined by general linear models with SAS software (Cary, North Carolina, USA).

*Results*

*Gross Lesions and Histopathology*

Only the athymic mice had macroscopical lesions. These animals all showed marked hepatomegaly, with multiple white foci (1 mm in diameter), marked splenomegaly, mild small
intestinal mural thickening, and mesenteric lymph nodes that were 1.25 times the size of control lymph nodes.

Microscopically, all of the euthymic mice had a granulomatous hepatitis, the granulomas affecting the majority of portal areas (Fig. 1A). The granulomas consisted of a small core of large macrophages surrounded by lymphocytes, plasma cells and fibrous connective tissue. Small inflammatory aggregates composed primarily of macrophages were also seen in the spleen, the lamina propria of the duodenum, and gut-associated lymphoid tissue (GALT). Euthymic control mice had no lesions.

Microscopically, all athymic mice had a severe granulomatous hepatitis, with large granulomas scattered throughout the parenchyma and within portal areas (Fig. 1B). Image analysis of the liver (Fig. 2) revealed that the lesional area, expressed as a proportion of the total area, was significantly ($P<0.01$) greater in athymic than in euthymic mice. The granulomas consisted primarily of numerous large macrophages surrounded by low numbers of neutrophils, occasional lymphocytes, and small amounts of fibrosis. Large numbers of macrophages and occasional neutrophils expanded the lamina propria and GALT in all sections of intestine. The duodenum was the most severely affected portion of the intestine, and the colon the least. Small intestinal villi were frequently widened and blunted. Semiquantitative scores of the duodenal lesions were significantly ($P<0.01$) greater in athymic than in euthymic mice, the mean scores being 4.2 and 1.5, respectively. Large numbers of macrophages were also seen in the spleen and pancreas. Athymic control mice had no lesions.

**iNOS Immunohistochemistry and Acid-fast Staining**

Within the euthymic mouse hepatic granulomas, the majority of macrophages showed intense iNOS labelling (Fig. 3A). In contrast, the majority of athymic mouse hepatic granuloma macrophages showed little or no labelling (Fig. 3B). Macrophages in granulomas with low numbers of lymphocytes and neutrophils typically showed the weakest labelling. The iNOS labelling of hepatic granulomas was significantly ($P<0.02$) greater in euthymic than in athymic mice (Fig. 4). Within inflammatory
foci, the macrophage was the only cell type that showed strong iNOS immunolabelling. There was very weak labelling by both iNOS antiserum and normal rabbit IgG (negative control) of intestinal epithelium, smooth muscle, peripheral ganglia, faecal material and proteinaceous fluid. Control mice showed no significant labelling.

Acid-fast staining of granulomas was significantly greater ($P<0.01$) in athymic than in euthymic mice (Fig. 4). Hepatic macrophages of athymic mice typically contained innumerable bacilli (Fig. 3B inset) while those of euthymic mice contained low numbers (Fig. 3A inset). There was no significant correlation ($P>0.05$) between iNOS labelling and acid-fast staining when individual granulomas were compared within each strain of mouse. Control mice showed no acid-fast staining.

**Electron microscopy**

In the liver of euthymic mice, macrophages containing bacteria were enlarged by numerous primary and secondary lysosomes. Most secondary lysosomes contained a mixture of amorphous electron-dense material and homogeneous electron-lucent material. Bacteria were either degraded and within secondary lysosomes, or intact and individually enclosed within a phagosome. There were also increased amounts of Golgi saccules adjacent to the nuclei (Fig. 5A).

In livers of athymic mice, macrophages containing bacteria were distended with numerous phagosomes, each containing a single intact bacterium. Only rarely did phagosomes contain multiple bacteria, degraded bacteria, or lysosomal, electron-dense fine granules. There were low numbers of primary lysosomes (Fig. 5B). Macrophages within the intestine had a similar appearance. Hepatic macrophages in euthymic mice differed from those in athymic mice in containing a higher percentage of degraded bacteria ($P<0.01$) (Fig. 6), fewer intact bacteria ($P<0.01$) and increased numbers of primary lysosomes ($P<0.04$) (Fig. 7).
Discussion

This study of euthymic and athymic mice infected with *M. a. paratuberculosis* demonstrated that the athymic animals were more susceptible and possessed macrophages with a lower degree of activation. Macrophage activation is defined as an increase in antimicrobial activity (North, 1978) and is associated with increased iNOS (Adler et al., 1996) and increased numbers of lysosomes (Cheville, 1994). In the present study, T cell-dependent macrophage activation was associated with increased resistance to *M. a. paratuberculosis*. Previous studies have shown that CD4 positive T cells and the associated cytokine IFN-γ were important in cell-mediated immunity against *M. avium* (Appelberg et al., 1994). Others have suggested that CD4 and CD8 cells are not an important part of the immune response to *M. a. paratuberculosis* infection (Adams et al., 1993).

In our experiment, the lower concentrations of iNOS in the T-cell deficient athymic mice than in the euthymic counterparts were associated with increased susceptibility to *M. a. paratuberculosis*. However, the T-cell deficiency probably also contributed to the increased susceptibility in numerous additional ways. Previous studies on mycobacterial infections have shown that both beneficial and detrimental effects may be attributable to iNOS. Genetically modified mice that lack the ability to produce iNOS, or "iNOS knockout" mice, have contributed greatly to the understanding of this substance. Thus, the mortality produced by the BCG strain of *Mycobacterium bovis* was greater in iNOS knockout mice than in control wild type mice (Garcia et al., 2000); the increased mortality was associated with increased numbers of bacteria and an exaggerated cellular immune response, characterized by increased tissue necrosis, increased interleukin-12 and increased tumor necrosis factor-alpha. Surprisingly, however, iNOS knockout mice were more resistant than control mice to *Mycobacterium avium* infection (Gomes et al., 1999), showing fewer bacteria, increased IFN-γ, and increased granuloma formation. In the *M. avium*-infected iNOS knockout mice, it would appear that the beneficial effects caused by the loss of nitric oxide immunoregulation outweighed the detrimental
effects associated with loss of nitric oxide bactericidal activity. In our study, low concentrations of iNOS were associated with increased susceptibility.

Increased numbers of primary lysosomes and increased bacterial degradation were observed in the more resistant euthymic mice. This was consistent with a study of *M. a. paratuberculosis* by Tanaka *et al.* (1996), who found that C3H mice, which are genetically more resistant than BALB/c mice, showed higher concentrations of acid phosphatase (a marker for lysosomes) and smaller numbers of bacteria. INOS knockout mice infected with *M. bovis* had reduced concentrations of acid phosphatase (Garcia *et al.*, 2000) which accords with our finding that low concentrations of iNOS were associated with reduced numbers of primary lysosomes.

The lesions of *M. a. paratuberculosis* in BALB/c mice are similar to those of bovine paratuberculosis, suggesting that this mouse strain is potentially useful as a model of the bovine disease. It seems likely that iNOS plays some role in Johne’s disease in cattle, since the production of nitric oxide has been demonstrated in bovine monocytes (Zhao *et al.*, 1997) and bovine macrophages (Jungi *et al.*, 1999) infected with *M. a. paratuberculosis*. However, bovine and murine macrophages regulate iNOS production differently *in vitro* (Adler *et al.*, 1995). Lipopolysaccharide stimulated both murine and bovine macrophages to produce iNOS, but only murine macrophages produced iNOS in response of IFN-γ (Adler *et al.*, 1995). Future studies clarifying these differences between murine and bovine iNOS regulation will strengthen the understanding and potential value of the mouse model of *M. a. paratuberculosis* infection.

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References


Fig. 1a,b. Representative liver sections from euthymic (a) and athymic (b) BALB/c mice infected with *M. a. paratuberculosis*. (a) Granulomas were composed of lymphocytes, plasma cells, neutrophils, and small amounts of fibrous connective tissue, centred on a few macrophages. (b) Granulomas had macrophages in the centre, and low numbers of neutrophils and lymphocytes at the periphery. Occasional inflammatory aggregates had few macrophages and large numbers of neutrophils. HE. Bar. 30 μm.
Fig. 2. Percent area of hepatic parenchyma with granulomas in euthymic (□) and athymic (■) mice infected with *M. a. paratuberculosis*. Means ± SEM. Significant difference (*P*<0.01).
Fig. 3a,b. Liver sections from euthymic (a) and athymic (b) BALB/c mice infected with *M. a. paratuberculosis*. (a) Immunoreactivity to iNOS was intense in macrophages. (b) Immunoreactivity to iNOS was weak in a few macrophages, but usually absent. Streptavidin-biotin-peroxidase method, haematoxylin counter stain. Inset: acid-fast stain. Bar, 30 μm.
Fig. 4. Percent surface of hepatic granulomas with iNOS labelling and acid-fast staining in euthymic (■) and athymic (■) mice infected with *M. a. paratuberculosis*. Means ± SEM.

*Significant difference (*P*<0.02).
Fig. 5a,b. Ultrathin liver sections from euthymic (a) and athymic (b) BALB/c mice infected with 

*M. a. paratuberculosis.* (a) Degraded bacteria (arrow) within a secondary lysosome. Multiple secondary lysosomes (arrowheads). (b) Single intact bacteria in phagosome (arrow) and multiple primary lysosomes (arrowheads). Uranyl acetate and lead citrate. TEM. Bar: 0.5 μm.
Fig. 6. Percent degraded bacteria within hepatic macrophages in euthymic (■) and athymic (□) mice infected with \textit{M. a. paratuberculosis}. Means ± SEM. Significant difference ($P<0.01$).
Fig. 7. Ultrastructural characteristics within hepatic macrophages in euthymic (□) and athymic (■) mice infected with *M. a. paratuberculosis*. Means ± SEM. *Significant difference (*P*<0.05).
CHAPTER 4: IMMUNOLOGIC RESPONSES OF CALVES FROM COWS SUBCLINICALLY INFECTED WITH MYCOBACTERIUM AVIUM SUBSPECIES PARATUBERCULOSIS

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Abstract

Objective—Determine the effects of maternal infection with M. a. paratuberculosis on the immunological responses of the offspring.

Animals—Seven pairs of calves and cows.

Procedure—Newborn calves from dams subclinically infected with M. a. paratuberculosis and calves from non-infected dams were given either 1x10^10 M. a. paratuberculosis or saline orally. Monthly, blood samples were collected and stimulated with M. a. paratuberculosis, M. avium, and M. bovis antigens and assayed for interferon gamma (IFN-γ) production. Calves were also tested for Johne's disease by serology, fecal culture and intradermal skin testing. The calves were euthanized and necropsied at 7 months of age.

Results—Calves from dams subclinically infected with M. a. paratuberculosis had significantly higher IFN-γ responses to M. avium and M. a. paratuberculosis antigens, prior to inoculation with either bacteria or saline on day 1, than did calves from non-infected dams. Cumulatively throughout the experiment, calves from dams subclinically infected with M. a. paratuberculosis had significantly higher IFN-γ responses to M. avium and M. bovis antigens compared to calves from non-infected dams. Two calves from infected dams had positive skin test responses and M. a. paratuberculosis isolated from tissues at the end of the experiment.

Conclusions and Clinical Relevance—Maternal infection with M. a. paratuberculosis resulted in a significant increase in IFN-γ production to mycobacterial antigens by their calves, as compared to
calves from non-infected dams. These results suggest that calves from infected dams may be identified early in life by their increased IFN-γ production to mycobacterial antigens.

Introduction

*Mycobacterium avium* subspecies *paratuberculosis* causes Johne's disease, a chronic granulomatous enteritis of cattle, sheep, and other ruminants. Clinical disease is typically seen in adult cattle, and consists primarily of weight loss and diarrhea. Affected cattle are afebrile and have a normal appetite despite progressively worsening diarrhea and malabsorption that eventually lead to death in several weeks to months after the onset of clinical signs. The disease is endemic in many countries and approximately 22% of U.S. dairy herds are infected with *M. a. paratuberculosis* resulting in estimated losses of $222 million per year.

Infection with *M. a. paratuberculosis* typically begins early in life through in-utero transmission, or more commonly through the ingestion of infective fecal material, colostrum, or milk. After infection, there is a prolonged subclinical stage which is characterized by intermittent fecal shedding of the bacteria, a partially protective cell mediated immune response, and a weak humoral immune response. Subclinical infections are difficult to detect using current tests, however, methods evaluating cell mediated immune responses appear to have the greatest potential to improve detection of subclinically infected cattle. As the infection progresses into clinical disease, cell mediated immune responses, as measured by interferon gamma (IFN-γ) production by peripheral blood monocytes or delayed-type hypersensitivity responses, decline. Concurrently there are increases in the humoral immune response and fecal shedding of bacteria. However, it has been recently shown that the increased humoral immune response seen in clinical disease is antigen and antibody isotype specific, and the humoral immune response to some antigens actually decreases in animals with clinical disease.

The effects of maternal infection with *M. a. paratuberculosis* on neonatal immunity and the outcome of fetal infection are largely unknown. Previous studies have shown that between 8.6 to
37% of fetuses from cows with Johne's disease are infected with *M. a. paratuberculosis*, and cows shedding large numbers of bacteria in their feces are the most likely to have in-utero infected calves.\(^4\) Maternal infection and exposure to mycobacterial antigens have been shown to alter neonatal immunity and result in antigen specific IFN-\(\gamma\) production in the human neonate.\(^13\) Similarly, bovine fetuses are also capable of developing antigen specific immune responses after exposure to *M. bovis*.\(^14\) Therefore, it was hypothesized that calves from infected dams have greater cell mediated immune responses to *M. a. paratuberculosis*, which in turn could alter susceptibility to *M. a. paratuberculosis* inoculation, compared to calves from non-infected dams. The objectives of the study were to compare the immune responses of calves from subclinically infected dams and calves from non-infected dams using newly developed and commercially available methods to detect Johne's disease.

**Materials and Methods**

**Experimental design**—Seven calves were grouped based upon infection status of their dam. Within each group, calves were randomly assigned to receive orally either *M. a. paratuberculosis* inoculum or saline daily for the first 4 days of life (Table 1). Blood and fecal samples were collected monthly throughout the experiment and the calves were euthanized and necropsied at 7 months of age. The care and use of the calves was approved by the Iowa State University Committee on Animal Care.

**Animals**—Calves 1-3 were from cows that had previously tested positive for *M. a. paratuberculosis* by fecal culture 1 to 3 times over a 2 year period, but were clinically normal. Calves 4-7 were from cows that were: negative for *M. a. paratuberculosis* by serology and fecal culture, originated from a herd in which all adult females were fecal culture negative, and the herd had no known cases of Johne's disease in the previous five years. Calves 1, 4-7 were Holsteins and 2-3 were Brown Swiss. Calves were removed from their dams at birth, given colostrum from their dam on the first day of life, and housed individually indoors.
Inoculum—The *M. a. paratuberculosis* inoculum originated from the ileal-cecal lymph node of a naturally infected cow with clinical Johne's disease. The bacterium was confirmed to be *M. a. paratuberculosis* by culture characteristics and PCR for IS900 gene. The bacteria was initially grown on Herrold's egg yolk medium slants supplemented with mycobactin J and incubated at 37°C. The bacteria was then transferred to Middlebrook 7H9 broth supplemented with 2 mg/liter mycobactin J. 10% OADC-BBL medium supplement and 2 ml/liter of glycerol.

Bacteria were concentrated by centrifugation (2000X g for 15 minutes) and re-suspended in 0.9% NaCl solution. Concentration of bacteria was determined by absorbance at 540 nm. Aliquots of 1x10^10 bacteria were frozen in saline/glycerol, stored at -70°C until used. Viability was determined by growth on Middlebrook 7H10 plates. Calves were given either 1x10^10 bacteria mixed in 10ml of saline or 10ml of only saline, followed by 30 cc whole milk, orally for the first four days of life.

Collection of samples—Whole blood was collected in sodium heparin for the IFN-γ assay. Serum was collected for ELISA testing, and feces was collected for bacterial culture from calves at the start of the experiment and at monthly intervals until the end of the experiment at 7 months of age. The last blood samples were collected three days prior to necropsy. An additional serum sample was collected on day 4 for ELISA testing to evaluate passive antibody transfer. Blood, serum, feces, along with colostrum, were also collected from the dams (except for dam 5) at parturition.

Bacterial cultures—Fecal samples were processed using the sedimentation method before being inoculated on Herrold's egg yolk medium slants with and without mycobactin J. Hexadecylpyridinium chloride solution (HPC) 0.9% was used to decontaminate the fecal sample. Tissue samples were decontaminated and homogenized in 0.75% HPC (2.5ml per 100mg tissue) prior to being inoculated on slants. Inoculated slants were incubated at 37°C and were evaluated for growth at 1-2 weeks after inoculation and at monthly intervals for 32 weeks.
Serology—Serum samples were frozen at -70°C until tested at the end of the experiment. Serum antibody to *M. a. paratuberculosis* was evaluated using a commercially available ELISA according to the manufacturer's instructions.  

Interferon gamma—One ml of whole blood was incubated with 10 μg of a whole cell sonicate of *M. a. paratuberculosis* strain 19698, *Mycobacterium avium* purified protein derivative (PPD), or *M. bovis* PPD for 18 hours at 39°C. Samples incubated with PBS served as nonstimulated controls. An aliquot of each sample was also incubated with concanavalin A and pokeweed mitogen to assure lymphocyte viability. Plasma was collected by centrifugation and frozen at -20°C until analyzed. Bovine IFN-γ levels in the plasma were measured with a commercially available ELISA according to the manufacturer's instructions and optical densities were measured with a microplate reader at a wavelength of 450nm. Data are presented as an absorbance index which was calculated by dividing the absorbance values of the antigen stimulated cells by the absorbance value of the non-stimulated cells.  

Skin testing—Calves were evaluated for a delayed type hypersensitivity response by intradermal skin tests when the calves were 1 day old, 4 months old, and 3 days prior to necropsy at 7 months of age. A novel *M. a. paratuberculosis* strain 19698, PPD was compared to USDA Johnin PPD. One tenth of one ml (0.1mg) of each PPD were injected intradermally and the site was measured with calipers 72 hours after the injection. If the 72 hour measurement was greater than 5mm thicker than the initial measurement, the test was considered positive.  

Necropsy procedure—Calves were euthanized by an intravenous injection of pentobarbital on day 210 of the experiment. The following tissues were collected for bacterial culture and histopathology: duodenum and pancreaticoduodenal lymph node; proximal, middle and distal jejunal segments and mesenteric lymph nodes; proximal, middle and distal ileal segments and mesenteric lymph nodes; ileocecal valve and ileocecal lymph node; cecum; proximal colon and colic lymph node; liver and hepatic lymph node; spleen; and mandibular lymph node.
Histopathology—Tissues were fixed in 10% neutral buffered formalin, routinely processed, embedded in paraffin and sectioned. Five micron sections were stained with H&E and the Ziehl-Neelsen technique to identify acid-fast bacteria. Immunohistochemistry to detect mycobacterial antigens was performed using a rabbit anti-\textit{M. bovis} antibody. Briefly, the sections were deparaffinized, rehydrated, and then simmered in a basic antigen retrieval solution. Sections were blocked with 20% normal goat serum and 3% bovine serum albumin followed by the primary antibody diluted 1:50,000 for one hour. Additional blocking with 2% hydrogen peroxide, 20% normal goat serum, and 3% bovine serum albumin preceded incubation with 1:400 diluted biotinylated goat anti-rabbit secondary antibody. Peroxidase conjugated streptavidin and NovaRED substrate were then applied. The slides were counterstained with hematoxylin. Negative controls substituted normal rabbit IgG as the primary antibody.

Statistical analysis—Means were tested by factorial and repeated measures ANOVA for the sources of variation under consideration. Results were considered significant at $P<0.05$.

Results

Interferon gamma—Calves from dams subclinically infected with \textit{M. a. paratuberculosis} repeatedly had higher IFN-\(\gamma\) responses throughout the experiment to all of the mycobacterial antigens, although the differences were not always statistically significant (Fig 1). On day 1 of the experiment, before the calves were inoculated with bacteria or saline, IFN-\(\gamma\) responses to \textit{M. avium} ($P<0.05$) and \textit{M. a. paratuberculosis} ($P<0.04$) antigens were significantly higher in calves from infected dams compared to calves of non-infected dams (Fig 2). When all time points of the experiment were combined, calves from infected dams had significantly higher IFN-\(\gamma\) responses with \textit{M. avium} ($P=0.02$) and \textit{M. bovis} ($P=0.001$) antigens than did the calves from non-infected dams (Fig 3).

The effect of oral inoculation with \textit{M. a. paratuberculosis} or saline was not significant within calves grouped by infection status of the dam (Table 2). However, when dam infection status was excluded, and calves were grouped solely on whether they were given \textit{M. a. paratuberculosis} or
saline orally, there was a significant difference. Calves inoculated orally with *M. a. paratuberculosis* (n=5) had significantly higher mean IFN-γ responses upon stimulation with both *M. avium* (*P=0.02*) and *M. bovis* (*P=0.001*) antigens than calves which were inoculated with saline (n=2) (Table 2). IFN-γ production was highest three months after inoculation with *M. a. paratuberculosis* for all three mycobacterial antigens.

There were no statistical differences or trends in IFN-γ production associated with the breed of the calves, and no significant differences in IFN-γ production were found between the infected and non-infected dams with any of the mycobacterial antigens at the time of parturition (data not shown).

**Serology**—All calves and cows were classified as negative for antibody to *M. a. paratuberculosis* during all time points of the experiment except for calf 1, which was positive on day 4.

**Bacterial cultures**—All monthly fecal cultures from the calves were negative for the duration of the experiment. At necropsy, the proximal ileum from calf 1, the proximal ileum from calf 2, and colonic lymph node from calf 2 were culture positive (Table 1). The dam of calf 1 was fecal culture positive on the day of parturition with 50 to 100 colonies of bacteria per slant. All other cows were fecal culture negative at parturition. Colostrum samples from all cows were also culture negative.

**Skin testing**—On day one of age, only the USDA Johnin PPD was administered to the calves and none of the calves had positive skin test reaction. At 4 months of age, only the novel *M. a. paratuberculosis* PPD was used and calves 1 and 2 had positive reactions with the remaining calves testing negative. At 7 months of age both PPDs were used, none of the calves had a positive skin test to the USDA Johnin PPD, and only calves 1 and 2 had positive reactions to the novel PPD with remaining calves having no response (Table 1).

**Histopathology**—There were no significant lesions, no acid fast bacteria, and no mycobacterial immunoreactivity present in any tissues from any of the calves.
Discussion

Subclinical infection with *M. a. paratuberculosis* of the dam resulted in a significant increase in IFN-γ production to mycobacterial antigens by their calves, when compared to calves from non-infected dams. The similar responses seen with all three of the stimulating mycobacterial antigens was likely due to the similarity of antigens among the mycobacterial preparations. The increased IFN-γ production may be explained by an in utero exposure to mycobacterial antigen. Human neonates born to tuberculin PPD sensitized mothers, had increased antigen specific IFN-γ production. Similarly, maternal trypanosomiasis, filariosis, and schistosomiasis have all been shown to alter antigen specific cytokine responses in the human neonate. Similarly in cattle, in utero exposure to *M. bovis* antigens have been shown to cause significant lymphocyte blastogenesis and delayed type hypersensitivity in bovine fetuses. Our day-one IFN-γ results, (prior to inoculation of the calves with mycobacteria or saline), which showed that calves from infected dams produced more IFN-γ to both the *M avium* and the *M. a. paratuberculosis* antigens compared to the calves from non-infected dams, are consistent with these previous studies. The increased IFN-γ levels that occurred throughout the experiment may also have been affected by increased antigen exposure after birth, since the only two calves that were culture positive made up two thirds of the calves within the infected dam group.

Skin testing may have elevated IFN-γ production in all calves and thus increased sensitivity of the assay. Whipple et al found that cattle skin tested with *M. bovis* and *M. avium* PPD had significantly increased IFN-γ responses for 3 to 28 days following skin testing. However since all calves were treated in the same manner, we believe that any increases due to skin testing would have occurred in all calves, and would not have affected our overall results.

Calves given bacteria had significantly increased IFN-γ responses to both the *M. avium* and *M. bovis* antigens cumulatively over the duration of the experiment, when our statistical analysis was
based solely on whether calves were given saline or *M. a. paratuberculosis* for the first four days of life. The increased IFN-γ response is consistent with other studies that have shown similar results upon natural or experimental *M. a. paratuberculosis* infections.¹⁹,²⁵,²⁶

The only two calves from which *M. a. paratuberculosis* was cultivated were from subclinically infected dams. These calves likely acquired the *M. a. paratuberculosis* by oral inoculation and not in utero, based on a previous study comprised solely of subclinically infected dams, which found 8.6% of fetuses were infected with *M. a. paratuberculosis*.¹ This finding may suggest that the calves from the infected dams were more susceptible to *M. a. paratuberculosis*, although the low number of calves in the study makes drawing any conclusions difficult. This conflicts with a larger field study in which calves from cows vaccinated with killed *M. a. paratuberculosis* and naturally infected with *M. a. paratuberculosis* were slightly less likely to become infected than calves from vaccinated uninfected cows.²⁷

The novel *M. a. paratuberculosis* PPD used for skin testing resulted in positive delayed type hypersensitivity response in only two calves, both of which were from infected dams and orally inoculated with *M. a. paratuberculosis*. The standard Johnin PPD skin tests were negative for all calves, which is consistent with a previous report which concluded the test was relatively insensitive.²⁸ Skin testing has long been a useful method to detect *M. bovis* in cattle, and this novel PPD may prove to be a useful test to detect exposure to *M. a. paratuberculosis* antigens, however our sample size is too small to verify this.²⁹

Footnotes

¹Allied Monitor. Fayette, MO.
²Difco Laboratories. Detroit, MI.
³Becton Dickinson, Sparks, MD.
⁴Sigma Chemical Co.. St. Louis, MO.
⁵IDEXX Laboratories Inc., Westbrook, ME.
Acknowledgments

The authors thank Dr. D. Jones, J. Gallup, T. Bosworth, and D. Moore for their valuable assistance.

References


| Infection status of Dam | Infected |  | Non-infected |  |
|------------------------|----------|----------------|-----------------|-----------------|-----------------|
| Calf treatment         | M. a. paratuberculosis | Saline | M. a. paratuberculosis | Saline |
| Calf ID number         | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| Summarized bacteriology results | pos | pos | neg | neg | neg | neg | neg |
| Summarized histopathological lesions | neg | neg | neg | neg | neg | neg | neg |
| DTH results with novel PPD | pos | pos | neg | neg | neg | neg | neg |
| DTH results with USDA Johnin | neg | neg | neg | neg | neg | neg | neg |

Table 1—Experimental design and necropsy results of calves from *M. a. paratuberculosis* infected dams or non-infected dams. Calves from each group were inoculated orally with wild type *M. a. paratuberculosis* or saline and necropsied at 7 months post-inoculation.
Figure 1—Interferon-γ mean absorbance indices of calves from *M. a. paratuberculosis* infected dams (white circles) compared to calves from non-infected dams (black circles) after stimulation with *M. a. paratuberculosis*, *M. avium*, and *M. bovis* antigens at monthly intervals. Absorbance index equals the optical density of antigen stimulated sample divided by the optical density of the non-stimulated sample. Mean ± SE.
Figure 2—Day 1 IFN-\(\gamma\) mean absorbance indices of calves from \textit{M. a. paratuberculosis} infected dams (white bars) compared to calves from non-infected dams (black bars) after stimulation with 3 different mycobacterial antigens. Absorbance index equals the optical density of antigen stimulated blood divided by the optical density of the non-stimulated blood. Mean ± SE calculated only from day 1 data. *Significant difference (\(P<0.05\)) between groups of calves.
Figure 3—Cumulative IFN-γ mean absorbance indices of calves from *M. a. paratuberculosis* infected dams (white bars) compared to calves from non-infected dams (black bars) after stimulation with 3 different mycobacterial antigens. Absorbance index equals the optical density of antigen stimulated blood divided by the optical density of the non-stimulated blood. Mean ± SE calculated from all sampled time periods over the duration of the experiment. *Significant difference (P<0.05) between groups of calves.
Table 2—Cumulative interferon γ mean absorbance indices of calves, from *M. a. paratuberculosis* (*M. a. ptb*) infected or non-infected dams, which were orally inoculated with *M. a. ptb* or saline.

Three different mycobacterial antigens were used to stimulate IFN-γ production from whole blood.

Absorbance index equals the optical density of antigen stimulated blood divided by the optical density of the non-stimulated blood. Mean ± SE calculated from all sampled time periods over the duration of the experiment. *Significant difference (P<0.05) between calves inoculated with *M. a. ptb* or saline.
CHAPTER 5: IMMUNOHISTOCHEMICAL AND ULTRASTRUCTURAL PATHOLOGY OF ILEUM IN BOVINE JOHNE'S DISEASE

A paper prepared for submission to Veterinary Pathology

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Abstract

To further understand the immunopathogenesis of bovine Johne's disease, sections of ileum from seven dairy cows with clinical Johne's disease and seven control cows were examined by immunohistochemistry. Further ultrastructural studies were performed on ileal tissues from the cows with Johne's disease. Morphometric analysis of digital images captured from light microscopy sections was used to quantify CD3 T cells, CD4 T cells, CD8 T cells, γδ T cells, CD79 B cells, and CD68 as a marker for macrophages. Immunoreactivity for inducible nitric oxide synthase (iNOS) and mycobacterial antigen was also accessed. The cows with Johne's disease had significantly greater immunoreactivity (P<0.05) for CD3 and γδ antigen within epithelial areas than control cows. While within the lamina propria the cows with Johne's disease had significantly greater immunoreactivity (P<0.05) for CD3, CD68, and mycobacterial antigen than control cows. Inducible nitric oxide synthase immunoreactivity occurred only in the cows with Johne's disease and was limited to macrophages and multinucleate giant cells located in the submucosa and serosa. Ultrastructurally, Mycobacterium avium subspecies paratuberculosis infected macrophages had a hypertrophied vacuolar transport system and numerous structurally intact bacilli that stained with immunogold labeled anti-mycobacterial antibody. The bacilli were located in heterogeneous bacteriophorous vacuoles that lacked characteristics typical of phagosomes and phagolysosomes. This study suggests that in clinical Johne's disease, γδ T cells are a significant component of the immune response within ileal villi and that the iNOS producing macrophages within the submucosa and serosa are functionally distinct from macrophages within the lamina propria which lacked iNOS immunoreactivity.
Introduction

*Mycobacterium avium* subspecies *paratuberculosis* (*M. a. paratuberculosis*) causes Johne's disease, a chronic enteritis of domestic ruminants, wild ruminants, and other species. Johne's disease is endemic in much of the world, and results in economic losses estimated to be over 1.5 billion dollars in the United States alone. Cattle in 22% of dairy herds and 7.9% of beef herds in the United States have evidence of *M. a. paratuberculosis* infection.

Johne's disease causes a granulomatous enteritis composed of numerous macrophages, epitheloid macrophages, multinucleate giant cells and variable numbers of lymphocytes. Ileal lymphocyte subsets have been examined in ovine Johne's disease and have shown increased γδ T cells in both experimental and natural disease. Blastogenesis studies of peripheral blood lymphocytes in bovine Johne's disease have shown that CD4 T cells, CD8 T cells, γδ T cells, and B cells all proliferate in response to mycobacterial antigens. These proliferative responses can be modified by the deletion or the introduction of other lymphocyte subsets into the assay suggesting an interaction between the different lymphocyte populations. The stage of the disease also affects lymphocyte proliferation as shown by a decrease in B cell proliferation in cows with clinical Johne's disease as compared to cows with subclinical disease. Mouse models of *M. a. paratuberculosis* infection have further defined the role of individual lymphocyte subsets and have shown the positive effects that T cells and CD4 T cells have on resistance to disease. Interestingly, γδ T cells appeared to have a negative affect on the immune response to *M. a. paratuberculosis* infection as shown by lower numbers of bacteria in the γδ T cell knockout mice as compared to wild type mice.

Inducible nitric oxide synthase (iNOS) is produce by macrophages and other cells to generate nitric oxide and additional reactive oxygen intermediates which have bactericidal and immunoregulatory effects. Bovine macrophages infected with *M. a. paratuberculosis* in vitro produce nitric oxide, but the role of nitric oxide in vivo is unclear. In other diseases of cattle, iNOS has been detected in lesions caused by *Arcanobacterium pyogenes, Mannheimia haemolytica*, and
Listeria monocytogenes. Studies of mycobacterial disease in iNOS knock out mice have shown the absence of iNOS is detrimental to the host in *M. tuberculosis* infections, but surprisingly the absence of iNOS appeared to be beneficial in *M. avium* infected iNOS knock out mice which had larger granulomas and fewer bacteria than the control mice.

The objectives of this study were to 1) quantify CD3 T cells, CD4 T cells, CD8 T cells, γδ T cells, CD79 B cells, CD68 cells, iNOS and mycobacterial antigen within the lamina propria and epithelial areas of ileal villi from cows with Johne's disease compared to control cows 2) describe the ultrastructural pathology of Johne's disease.

### Materials and Methods

#### Animals

All seven dairy cows with Johne's disease cows had historical and clinical examination findings of weight loss and diarrhea. All cows had positive results when tested for antibody to *M. a. paratuberculosis* using a commercially available ELISA following the manufacturer's instructions (IDEXX Laboratories Inc., Westbrook, ME). *M. a. paratuberculosis* was cultured from multiple different tissues from all cows. Sections of ileum and mesenteric lymph nodes were histopathologically consistent with Johne's disease, contained acid-fast bacteria, and mycobacterial antigen.

None of the seven control dairy cows had historical or clinical examination evidence of Johne's or other intestinal disease. *M. a. paratuberculosis* was not cultured from the proximal ileal, distal ileal or mesenteric lymph nodes of any control cows. There were no microscopic lesions consistent with Johne's disease, no acid fast bacilli or mycobacterial antigen detected in proximal ileal, distal ileal, and mesenteric lymph nodes samples from any of the control cows.

#### Histopathology and Immunohistochemistry

The ileum was sampled from all cows 30 cm proximal to the ileal-cecal junction and fixed in 10% neutral buffered formalin, routinely processed, and embedded in paraffin. Adjacent sections of
ileum were frozen at -70°C in Tissue-Tek O.T.C. Compound (Sakura Finetek U.S.A. Inc., Torrance, CA).

Formalin fixed tissues were stained with H&E, acid-fast Ziehl-Neelsen, and used for immunohistochemistry. For immunohistochemistry, three micron sections were stained by standard streptavidin-biotin immunoperoxidase methods to detect mycobacterial antigens, inducible nitric oxide synthase, and CD79 antigens. Mycobacterial antigens were detected using rabbit polyclonal anti-M. bovis antibody (Dako, Carpinteria, CA) diluted 1:50,000. A section of ileum from a cow previously diagnosed with Johne’s disease was used as a positive control. B-cells were detected using mouse monoclonal anti-human CD79 antibody (Dako) diluted 1:800 with a section of normal bovine lymph node used as a positive control. Inducible nitric oxide synthase was detected using rabbit polyclonal anti-mouse iNOS antibody (Upstate Biotechnology, Lake Placid, NY) diluted 1:10,000 following microwave heating in AR10 Basic Antigen Retrieval solution (BioGenex, San Ramon, CA). The positive control slide was a section of lymph node from a calf infected with M. bovis. Slides were counterstained with hematoxylin. Negative controls substituted normal sera (iNOS and M. bovis) or saline (CD79) as the primary antibody.

Frozen sections were used to detect CD3 T-cells, CD8 T-cells, γδ T-cells, CD4 T-cells, and CD68 cells. Four micron sections were applied to slides using the CryoJane Tape-Transfer System (Instrumedics Inc., Hackensack, NJ), fixed in -20°C ethanol for 8 minutes, and air dried at room temperature overnight.

Sections were blocked with 20% normal swine serum for 30 minutes followed by the primary antibodies for two hours at room temperature. The following primary antibodies and dilutions were used: mouse monoclonal anti-bovine CD3 diluted 1:1000 (VMRD Inc., Pullman, WA), mouse monoclonal anti-bovine CD8 diluted 1:1000 (Serotec, Raleigh, NC), mouse monoclonal anti-bovine γδ TCR diluted 1:1000 (VMRD), mouse monoclonal anti-bovine CD4 diluted 1:50 (VMRD), and mouse monoclonal anti-CD68 (human macrophage) (Dako). Endogenous peroxidase was quenched
with 0.3% H2O2 in methanol for 30 minutes, followed by 1:300 diluted biotinylated goat anti-mouse secondary antibody (KPL, Gaithersburg, MD). Slides were incubated with peroxidase conjugated streptavidin(BioGenex) for 35 minutes and then NovaRED substrate (Vector Labs, Burlingame, CA) for 2 minutes. The slides were counterstained with hematoxylin. Negative controls substituted normal mouse IgG as the primary antibody. Lymph node sections were used as positive controls.

**Image analysis**

Five random fields of ileal villi with appropriately stained internal controls were electronically photographed using a axiophot microscope (Carl Zeiss Inc., Thornwood, NY) with the 10x objective by 1.6x optivar, combined with a DXC-3000A color video camera (Sony, Itasca, IL), and a CG-7 frame grabber board (Scion, Frederick, MD). The lamina propria and epithelial areas were delineated from each other using an electronic graphics tablet and Photoshop 5.0 (Adobe Systems Inc., San Jose, CA). The edited images were analyzed using IPLab software (Scanalytics, Fairfax, VA). Immunohistochemical staining surface areas were quantified within the epithelium and lamina propria, and expressed as a percentage of the respective outlined area for each section.12

**Electron microscopy**

Sections of ileum were fixed in 2.5% glutaraldehyde for 4 hours and then post fixed in 1% osmium tetroxide or fixed in 4% osmium tetroxide for 3 hours. Tissues were then dehydrated and embedded in Spurr's resin (EMS, Fort Washington, PA). One micron sections were stained with toluidine blue, examined, and ultrathin sections were made of select areas. These sections were stained with uranyl acetate and lead citrate, and examined with a Phillips 410 transmission electron microscope (FEI Co., Hillsboro, OR). Immunoelectronmicroscopy was done on glutaraldehyde fixed tissue on nickel grids. Grids were etched with saturated sodium metaperiodate solution, rinsed, blocked with 1% bovine serum albumin, and incubated with rabbit polyclonal anti- \textit{M. bovis} antibody (Dako) diluted 1:10,000. The secondary antibody was gold labeled goat anti-rabbit (Ted Pella Inc., Redding CA) diluted 1:10.
Statistical analysis

Means, standard error of means, and sources of variation were determined by general linear models with SAS software (SAS Institute Inc., Cary, NC). Results were considered significant at \( P<0.05 \).

Results

Histopathology and immunohistochemistry

All 7 of the cows with Johne's disease had severe granulomatous or lymphoplasmacytic ileitis and lymphangitis with variable numbers of acid-fast bacteria. Four of the cows with Johne's disease had a severe granulomatous ileitis with numerous acid-fast bacteria. There were fewer macrophages, epitheliod macrophages, and intracellular acid-fast bacteria within the lesions of the remaining three cows. No significant lesions or acid-fast bacteria were within the ileal sections from the control cows.

The cows with Johne's disease had significantly greater immunoreactivity \( (P<0.05) \) for CD3 and \( \gamma\delta \) antigen within epithelial areas than did the control cows (Fig. 1). Within the lamina propria, the cows with Johne's disease had significantly greater immunoreactivity \( (P<0.05) \) for CD3, CD68, and mycobacterial antigen than did the control cows (Fig. 2). There were no significant differences between the cows with Johne's disease and control cows in either the lamina propria or epithelial areas for CD4, CD8, and CD79 immunoreactivity (Fig. 1, 2).

Immunoreactivity was concentrated in the epithelial areas for CD3, \( \gamma\delta \), and CD8 in both the cows with Johne's disease and the control cows (Fig. 3-8). CD4 immunoreactivity was concentrated within the lamina propria (Fig. 9, 10). Very little CD79 immunoreactivity was within the superficial mucosa areas that were quantified, but there was abundant staining within submucosal lymphoid follicles (Fig. 11, 12). There was a significant difference in CD68 immunoreactivity between the cows with Johne's disease and the control cows \( (P<0.03) \), and immunoreactivity was primarily in the lamina propria (Fig. 13, 14).
iNOS immunoreactivity was very sparse within the lamina propria and epithelial areas in both groups of cattle (Fig. 1.2,15,16). Infrequently there were focal areas of intense iNOS immunoreactivity in the submucosa of 3 cows with Johne's disease (Fig. 17). These areas of intense immunoreactivity were detected within macrophages and multinucleate giant cells that composed serosal and submucosal granulomas (Fig. 18a). Immunoreactivity for mycobacterial antigen on serial sections was frequently within these same inflammatory aggregates (Fig. 18b).

Mycobacterial immunoreactivity within the cows with Johne's disease was highly variable. Four of the cows had multibacillary lesions with abundant antigen and 3 of the cows had paucibacillary lesions with rare immunoreactivity. No mycobacterial immunoreactivity was detected in any of the control cows.

**Electron microscopy**

Macrophages, multinucleate giant cells and neutrophils contained intracytoplasmic bacilli. The cytoplasm of infected macrophages and giant cells were enlarged by a hypertrophied vacuolar transport system including: large (>1000nm) vacuoles and multivesicular bodies containing weakly electron dense flocculent material, small < 150nm clear vacuoles, bacteriophorous vacuoles, endoplasmic reticulum and golgi (Fig. 19). Mitochondria were prominent and there were few electron dense 100-300nm vesicles (lysosomes). Numerous filopodia extended from the cell membrane and interdigitated with surrounding cells. Low numbers of macrophages infected with numerous bacilli were shrunken and had a markedly electron dense cytoplasm and nucleus.

Structurally intact bacilli were in several types of vacuoles, with the majority of bacilli being singly within a tightly adherent vacuole. Less frequently bacilli were in clusters of two to fourteen bacilli in large, electron lucent, loosely adherent vacuoles. Multivesicular vacuoles and vacuoles filled with myelin whirls also contained bacilli (Fig. 20). Electron dense lysosomal like contents were rarely within phagosomes and few bacilli were fragmented or had irregular cell walls. Bacilli were
ultrastructurally compatible with mycobacteria and stained with immunogold labeled anti
mycobacteria antibody (Fig. 21).

Discussion

γδ T cells have been shown to increase in a variety of bovine diseases such as tuberculosis,
cryptosporidiosis, and coccidiosis. In this study we also determined that there were increased γδ
T cells within the ileum of cattle with Johne’s disease which is consistent with immunohistochemical
studies of sheep with Johne’s disease. Sheep with the tuberculoid form of Johne’s disease had
increased numbers of γδ T cells in ileal villi compared to control sheep, and sheep with lepromatous
lesions had a higher percentage of γδ T cells in the ileal villi then did control sheep. γδ T cells were
increased within ileal villi of lambs by four weeks after experimental inoculation with M. a.
paratuberculosis. Bovine in vitro studies and murine models of M. a. paratuberculosis infection
have suggested an immunoregulatory role for γδ T cells in M. a. paratuberculosis infections.
Peripheral blood γδ T cells from cattle infected and or sensitized to M. a. paratuberculosis inhibited
M. a. paratuberculosis antigen induced proliferation of CD4 T cells. This inhibition of CD4 T cells
by γδ T cells was blocked by the addition of CD8 T cells to the lymphocyte proliferation assay.
Similar results were seen in cattle infected with M. bovis. Depletion of γδ T cells from in vitro M.
bovis antigen induced proliferation of peripheral blood monocytes resulted in an increase in
proliferation of αβ T cells. γδ T cells also appeared to have a detrimental effect on the host in
studies of M. a. paratuberculosis infected γδ T knockout mice, in which the knockout mice had fewer
bacteria and smaller granulomas than infected wild type mice.

The lack of immunoreactivity for iNOS in the lamina propria is similar to previous in vitro
studies which saw only small amounts or no increase in nitric oxide production by mononuclear
phagocytes infected with M. a. paratuberculosis. The lack of iNOS immunoreactivity suggests a
lack of macrophage activation, even though many of the macrophages in our study contained
numerous intracellular bacilli. In contrast to this are macrophages within the submucosa and serosa that had both iNOS and *M. a. paratuberculosis* immunoreactivity. It is uncertain why this second, much smaller population of infected macrophages were producing iNOS. The macrophages producing iNOS in the submucosa and serosa may have had greater cytokine stimulation than those in the lamina propria. IFN-γ is a potent stimulator of macrophages and CD4 T cells produce significantly greater amounts of IFN-γ in response to mycobacterial antigen than CD8 T cells or γδ T cells in cattle infected with *M. a. paratuberculosis*. Concentrations of CD4 T cells vary depending on the location within the ileum, and there are increased CD4 T cells in the deeper gut associated lymphoid tissues such as the interfollicular areas compared to the more superficial mucosa.

This study's findings of a hypertrophied vacuolar transport system within infected macrophages agrees with previous in vivo and in vitro bovine studies. Most bacteriophorous vacuoles lacked evidence of lysosomal fusion which is consistent with a study of ovine Johne's disease in which macrophages with high numbers of intact bacilli had few lysosomes and phagolysosomes. Inhibition of lysosomal fusion with *M. a. paratuberculosis* containing vacuoles has also been shown to occur during in vitro studies of murine J774 macrophages.

We prefer the term bacteriophorous vacuole for vacuoles that contain a structurally intact bacteria that do not have the typical appearance of a phagolysosome. In vitro studies have shown strong evidence that *M. a. paratuberculosis* and other mycobacteria modify the development and maturation of the vacuole so that the bacteria can both survive and replicate. This is in distinction to endosomes and phagolysosomes which are degradative organelles and contain evidence of disintegration of the bacterial cell. The term is analogous to a parasitophorous vacuole, which is used primarily for intracellular protozoa, but has also been used to describe intracellular bacteria.
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References


29 NAHMS: Johne's disease on U.S. dairy operations. 1997


40 Veazey, R. S., Horohov, D. W., Krahenbuhl, J. L., Taylor, H. W., Oliver, J. L. and Snider, T. G.: 3rd: Differences in the kinetics of T cell accumulations in C3H/HeN (Bcg-resistant) and C57BL/6 (Bcg-susceptible) mice infected with *Mycobacterium paratuberculosis*. Comp Immunol Microbiol Infect Dis. 19:289-304. 1996


Fig. 1. Percent of ileal mucosa epithelial area with CD3, γδ (GD), CD8, CD4, CD68, iNOS, and mycobacterial (Mycob.) immunoreactivity. Mean ± standard error of the mean. *Significant difference (P<0.05).
Fig. 2. Percent of ileal lamina propria with CD3, γδ (GD), CD8, CD4, CD68, iNOS, and mycobacterial (Myco.) immunoreactivity. Mean ± standard error of the mean. *Significant difference ($P<0.05$).
Fig. 3. Ileum; cow with Johne's disease. Increased CD3 immunoreactivity within epithelium and lamina propria compared to control cow. Streptavidin-biotin-peroxidase method. Harris's hematoxylin counter stain. Bar = 110 μm.

Fig. 4. Ileum; control cow. CD3 immunoreactivity within epithelium and lamina propria. Streptavidin-biotin-peroxidase method. Harris's hematoxylin counter stain. Bar = 85 μm.

Fig. 5. Ileum; cow with Johne's disease. Increased γδ immunoreactivity within epithelium compared to control cow. Streptavidin-biotin-peroxidase method. Harris's hematoxylin counter stain. Bar = 106 μm.

Fig. 6. Ileum; control cow. γδ immunoreactivity concentrated within epithelium. Streptavidin-biotin-peroxidase method. Harris's hematoxylin counter stain. Bar = 77 μm.

Fig. 7. Ileum; cow with Johne's disease. CD8 immunoreactivity primarily within epithelium. Streptavidin-biotin-peroxidase method. Harris's hematoxylin counter stain. Bar = 94 μm.

Fig. 8. Ileum; control cow. CD8 immunoreactivity primarily within epithelium. Streptavidin-biotin-peroxidase method. Harris's hematoxylin counter stain. Bar = 85 μm.

Fig. 9. Ileum; cow with Johne's disease. CD4 immunoreactivity primarily within lamina propria. Streptavidin-biotin-peroxidase method. Harris's hematoxylin counter stain. Bar = 106 μm.

Fig. 10. Ileum; control cow. CD4 immunoreactivity primarily within lamina propria. Streptavidin-biotin-peroxidase method. Harris's hematoxylin counter stain. Bar = 94 μm.
Fig. 11. Ileum: cow with Johne's disease. CD79 immunoreactivity within epithelium and lamina propria was minimal. Streptavidin-biotin-peroxidase method. Harris's hematoxylin counter stain. Bar = 85 μm.

Fig. 12. Ileum: control cow. CD79 immunoreactivity within epithelium and lamina propria was minimal. Streptavidin-biotin-peroxidase method. Harris's hematoxylin counter stain. Bar = 85 μm.

Fig. 13. Ileum: cow with Johne's disease. Increased CD68 immunoreactivity within lamina propria compared to control cow. Streptavidin-biotin-peroxidase method. Harris's hematoxylin counter stain. Bar = 85 μm.

Fig. 14. Ileum: control cow. CD68 immunoreactivity was concentrated within lamina propria. Streptavidin-biotin-peroxidase method. Harris's hematoxylin counter stain. Bar = 77 μm.

Fig. 15. Ileum: cow with Johne's disease. iNOS immunoreactivity within epithelium and lamina propria was minimal. Streptavidin-biotin-peroxidase method. Harris's hematoxylin counter stain. Bar = 94 μm.

Fig. 16. Ileum: cow with Johne's disease. iNOS immunoreactivity within epithelium and lamina propria was minimal. Streptavidin-biotin-peroxidase method. Harris's hematoxylin counter stain. Bar = 85 μm.
Fig. 17. Ileum: cow with Johne’s disease. Marked iNOS immunoreactivity within submucosal and serosal inflammatory aggregates. Streptavidin-biotin-peroxidase method. Harris’s hematoxylin counter stain. Bar = 150 μm.

Fig. 18a. Ileum: cow with Johne’s disease. Marked iNOS immunoreactivity within submucosal macrophages and multinucleate giant cells. Streptavidin-biotin-peroxidase method. Harris’s hematoxylin counter stain. Bar = 100 μm.

Fig. 18b. Ileum: cow with Johne’s disease. Serial section of 18a. Mycobacterial immunoreactivity within the same submucosal inflammatory aggregates that had iNOS immunoreactivity. Streptavidin-biotin-peroxidase method. Harris’s hematoxylin counter stain. Bar = 100 μm.
Fig. 19. Ileum: cow with Johne's disease. Infected macrophage within the lamina propria that has a hypertrophied vacuolar transport system, prominent golgi (arrows), mitochondria (arrowheads), nucleus (n) and interdigitating cell membrane. Uranyl acetate and lead citrate. TEM. Bar = 1.56 μm.

Fig. 20. Ileum: cow with Johne's disease. Cytoplasm of macrophage within the lamina propria. Numerous heterogeneous bacteriophorous vacuoles with single intact bacilli and closely opposed vacuolar membrane (arrow), and bacilli in large vacuoles with membranous material (arrowheads) Uranyl acetate and lead citrate. TEM. Bar = 1.21 μm.
Fig. 21. Ileum: cow with Johne’s disease. Mycobacterial immunoreactivity within
bactenophorous vacuole and bacillus. Immunogold method. Uranyl acetate and lead citrate.

TEM. Bar = 0.21μm.
CHAPTER 6. GENERAL CONCLUSIONS AND FUTURE STUDIES

Conclusions

T cell and macrophage responses to *M. a. paratuberculosis* were studied in experimentally inoculated BALB/c mice, experimentally inoculated calves from cows infected with *M. a. paratuberculosis* and non-infected cows, and naturally infected adult cattle. In the study of BALB/c mice, the euthymic mice were less susceptible to *M. a. paratuberculosis* infection than the T cell deficient athymic nude mice as shown by smaller hepatic granulomas and decreased numbers of bacteria within the euthymic mice. It was concluded that macrophage activation was increased in the euthymic mice as evidenced by increased iNOS immunoreactivity, lower numbers of bacteria, an increased percentage of degraded bacteria, and increased numbers of primary lysosomes within infected macrophages.

In the second study of calves, no microscopic lesions consistent with *M. a. paratuberculosis* were detected, but there were significant differences in IFN-γ responses to mycobacterial antigens between calves from *M. a. paratuberculosis* infected cows and calves from non-infected cows. Calves from naturally infected cows had greater IFN-γ responses to mycobacterial antigens than calves from non-infected cows. This suggests that calves from *M. a. paratuberculosis* infected cows can be identified at an early age by their IFN-γ responses. The novel PPD used for the concurrent skin testing also performed well as it detected the only two calves that had *M. a. paratuberculosis* isolated from tissues.

The final study examined ileal villi from cows with clinical Johne’s disease compared to cows with no evidence of *M. a. paratuberculosis* infection. The cows with Johne’s disease had increased CD3 immunoreactivity in epithelial areas and the lamina propria, increased γδ immunoreactivity in epithelial areas, increased CD68 immunoreactivity in the lamina propria, and increased mycobacterial antigen immunoreactivity within the lamina propria as compared to control cows. This shows for the first time that intestinal γδ T cells are a significant component of the T cell
response in bovine Johne’s disease. Inducible nitric oxide synthase immunoreactivity was limited to macrophages in the submucosa and serosa from cows with Johne’s disease. This suggests that the iNOS producing macrophages within the submucosa and serosa are functionally distinct from the macrophages within the lamina propria which lacked iNOS immunoreactivity. Ultrastructurally, \textit{M. a. paratuberculosis} infected macrophages had a hypertrophied vacuolar transport system and numerous structurally intact bacilli. The bacilli were located in vacuoles that had a variety of different characteristics. The study concluded that bacteriophorous vacuole was an appropriate term for these vacuoles because they did not have characteristics typical of phagosomes and phagolysosomes.

\textbf{Future Studies}

Based on the findings of the second study that involved calves, it would be interesting to pursue the effects of maternal disease on the offspring’s immune responses along two different avenues. First, continue to compare calves from \textit{M. a. paratuberculosis} infected cows to calves from non-infected cows, but modify the immunological challenge which was previously an oral inoculation of \textit{M. a. paratuberculosis}. Oral inoculation is a difficult and frequently unsuccessful method to challenge the immune system and takes many months to evaluate the calves’ response. Using a parenteral inoculation of \textit{M. a. paratuberculosis} would be a more rapid and repeatable method to challenge and access the calf’s immune responses. The primary hypothesis would be that calves from \textit{M. a. paratuberculosis} infected dams eliminate a subcutaneous challenge of \textit{M. a. paratuberculosis} from the injection site and draining lymph nodes more rapidly than calves from normal dams.

A related project would be to examine the effects of bovine maternal infection with helminthes on the immune responses of the calf. The human studies showed that maternal infections alter the neonatal immune responses and that maternal helminth infections slant the infant’s immune system toward the Th-2 response. When infants were challenged by BCG vaccination and than
tested for IFN-γ response at one year of age, children from parasitized mothers made less IFN-γ then similarly treated infants from non-parasitized mothers. This would likely make the infants from parasitized mothers more susceptible to diseases requiring a Th-1 response such as mycobacterial diseases. The hypothesis would be that calves from cows free of helminth parasites have greater Th-1 immune responses (as shown by greater IFN-γ responses to mycobacterial antigens and lipopolysaccharide) then calves from dams with helminth parasites. This would imply two things: that calves from parasitized cows would be more susceptible to diseases that require a Th-1 immune response, and that deworming cows, which is expensive and often not done by the producer to save money, has newly discovered health benefits for the calf. Obviously to prove the two implications stated above, it would take a series of experiments. But if found to be true, the results would be a great benefit to cattle producers and be a useful marketing tool for pharmaceutical companies.

The results of the third study of cows with Johne’s disease opens up a whole series of questions that would be fascinating to investigate. The differences observed in the iNOS immunoreactivity between infected macrophages in the lamina propria and infected macrophages in the submucosa of cows with Johne’s disease suggest there are two functionally distinct populations of macrophages. Why do some macrophages in the submucosa produce iNOS while none of the numerous macrophages in the lamina propria produce iNOS? To begin to answer this question one could compare the lymphocyte populations in those two distinct areas by immunohistochemistry. Once the lymphocyte populations were defined, then the local cytokine levels could be evaluated. This could be done by comparing the levels of IFN-γ and IL-4 from lymphocytes and IL-1 and IL-12 from macrophages between the two sites by immunohistochemistry and measuring mRNA from cells collected by laser capture in those specific areas. IFN-γ and IL-12 have already been shown to have significant effects on the outcome of M. avium infections, so it is probable that they will have a significant impact on M. a. paratuberculosis infections. It is conceivable that there is a Th-1 like response in the submucosa and Th-2 like response in the lamina propria. Perhaps the submucosal
lesions, which are likely more recent infections, are comparable to the early subclinical stage of Johne's disease in which Th-1 responses dominate? Perhaps the lamina propria infections are of longer duration and are comparable to the Th-2 immune responses seen in clinical Johne's disease? In any case, it does appear that in-vivo *M. a. paratuberculosis* infected macrophages behave differently based on the anatomic location. All of these techniques discussed previously for the ileum could then be used to evaluate mesenteric lymph nodes. In caprine Johne's disease, increased numbers of CD8 T cells and decreased numbers of CD4 T cells within the paracortex of mesenteric lymph nodes has already been shown occur, so similar changes likely occur in cattle.\(^7\)

Another set of questions worth investigating centers on macrophage activation and what effects does this have on the bacilli? Perhaps the iNOS immunoreactive macrophages observed in the submucosa also produce more reactive oxygen intermediates, lysozymes and have more acidic vacuoles.\(^5\) More importantly, are these iNOS immunonreactive macrophages more effective in killing *M. a. paratuberculosis*? There is evidence that the bacilli have developed a defense mechanism against free radicals. *M. a. paratuberculosis* and other virulent mycobacteria have been shown to produce superoxide dismutases which block reactive oxygen intermediates and thus block the formation of peroxynitrite.\(^8,9\)

What happens when the fact that bovine macrophages in the lamina propria lack of iNOS immunoreactivity is considered in the search for the optimum mouse model?\(^5\) Perhaps the best mouse model to mimic conditions in the bovine lamina propria is an iNOS knockout mouse? Using this model would also increase the understanding of the affects of iNOS on *M. a. paratuberculosis* in vivo.

A final potential study would be to characterize the numerous intracytoplasmic vesicles within ileal *M. a. paratuberculosis* infected bovine macrophages that are not seen in hepatic *M. a. paratuberculosis* infected murine macrophages.\(^5,10\) Many of the bacteriophorous vacuoles present in cattle also have a different appearance than those in mice.\(^5,10\) Understanding the differences in these
vacuoles between the two species may further the understanding of the intracellular pathogenesis of *M. a. paratuberculosis*.

References


