Characterization of the Cellular Immune Defect in Chronic Bovine Paratuberculosis.

John Michael Kreeger
Louisiana State University and Agricultural & Mechanical College

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CHARACTERIZATION OF THE CELLULAR IMMUNE DEFECT IN
CHRONIC BOVINE PARATUBERCULOSIS

A Dissertation
Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Interdepartmental Program
in Veterinary Medical Sciences
Veterinary Pathology

by
John Michael Kreeger
D.V.M., Louisiana State University, 1984
May 1988
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ABSTRACT

Cattle, naturally infected with *Mycobacterium paratuberculosis*, were evaluated with respect to immune status. Their ability to express cell mediated immune responses to homologous and heterologous antigens was also evaluated. Lymphocyte and macrophage augmentation was attempted to localize the immune defect in cattle with paratuberculosis.

Infected cattle had varying responses to johnin skin test antigen. Lymphocyte blastogenic responses and migration inhibition were, however, consistently positive in infected cattle. Non-infected cattle had positive skin test reactions, blastogenic responses, and migration inhibition after sensitization with *M. bovis* BCG, *M. paratuberculosis*, and keyhole limpet hemocyanin. No changes were seen in lymphocyte blastogenic response, migration inhibition, interleukin-2 production, and interleukin-1 release in infected cattle after sensitization.

Rifabutin treatment increased cutaneous hypersensitivity to johnin skin test antigen. No lesion alterations were induced with this antimicrobial agent.
Preliminary evidence indicated that blood monocytes phagocytize bacteria and take up ferritin. It could not be accurately determined if killing of the organism had taken place. The ferritin was seen in round clumps suggestive of secondary lysosomes and as small grains in association with bacteria. This may have represented phagosome-lysosome fusion. Interleukin-2 production was decreased in infected cattle from that in non-infected controls. Non-infected cattle significantly increased interleukin-2 production in response to sensitizing antigens while no effect was seen in the infected animals.

Transfer factor specific for *M. paratuberculosis* and keyhole limpet hemocyanin augmented cell-mediated responses in infected cattle as evidenced by positive migration inhibition and increased interleukin-2 production. No alterations in ileal and mesenteric lymph node lesions were noted after transfer factor treatment.

Blood monocytes from infected cattle spontaneously released high levels of interleukin-1 and increased release with bacterial lipopolysaccharide stimulation. Non-infected cattle released significant levels with keyhole limpet hemocyanin, *M. bovis* PPD, and johnin with the highest release occurring with johnin stimulation.
Interleukin-1 release was not affected by sensitization with \textit{M. bovis} BCG, \textit{M. paratuberculosis} and keyhole limpet hemocyanin.
CHAPTER 1

LITERATURE REVIEW AND OBJECTIVES

General

Bovine paratuberculosis is a chronic debilitating enteric infection of ruminants caused by the acid-fast bacillus *Mycobacterium paratuberculosis*. The disease was first described by Johne and Frothingham in 1895, \(^1\) hence the name, Johne's disease. The organism was first isolated by Twort in 1910\(^2,3\) and became named *Mycobacterium enteriditis chronicae pseudotuberculosaebovis johne*. The disease later became known as paratuberculosis or Johne's disease and the causative agent became *Mycobacterium paratuberculosis*.

Paratuberculosis occurs throughout the world and has a relatively wide host range. In addition to sheep and goats, numerous species of wildlife,\(^4,5,6,7,8,9,10,11\) exotic animals,\(^12,13,14,15,16,17,18,19,20\) and non-conventional domestic animals\(^17,21,22\) have been infected. Monogastrics may also become infected under special circumstances.\(^23-28\) The organism may multiply in these hosts, but rarely results in clinical disease. The implication remains, however that these hosts may serve
as a reservoir for ruminant infection.

Paratuberculosis was first described in North America in 1908\textsuperscript{29} and has since been observed in virtually every state. A 1983 abattoir survey of 1000 cattle in Wisconsin showed histologic lesions of Johne's disease in 11% of the animals examined.\textsuperscript{30} In a New England-based study a prevalence rate of 18% was shown by employing culture techniques in addition to histologic evaluation.\textsuperscript{31} It was concluded that histologic evaluation alone was too inaccurate and insensitive. In a recent nationwide survey of 32 states and Puerto Rico, Merkal et al\textsuperscript{222} examined lymph node specimens from 7,540 animals by culture methods and found an overall prevalence of bovine paratuberculosis of 1.6%. The prevalence in dairy and beef cattle was determined to be 2.9% and 0.8%, respectively. It has been estimated that losses for the dairy industry in the United States exceed $1.5 billion per year.\textsuperscript{32}

Animals are usually infected at less than 30 days of age and evidence by experimental inoculation suggested that cattle must become infected during calfhood in order to develop clinical disease because of the development of age-related resistance.\textsuperscript{33-43} It is estimated that only one-third of the exposed animals ever become infected.\textsuperscript{44} Development of disease is dependent on the size of the exposure dose and the immunologic status of the host.\textsuperscript{44}
Animals infected as adults may develop less obvious lesions or eliminate the organism. Experimentally infected animals have been reported to recover. Some animals exposed to the organism never develop clinical disease, but may become carriers and shedders of the organism.

The Channel Island and Shorthorn breeds appear to have the greatest incidence of infection among cattle. In Louisiana, however, the Brahman is frequently infected. This may be simply due to the larger number of Brahman and Brahman-cross cattle in Louisiana versus Channel Island breeds.

Ingestion of feces containing high number of bacteria is the primary source of infection for the neonate. Once ingested the organism penetrates the intestinal mucosa through Peyer's patches. The organism is then phagocytized by macrophages and survives intracellularly where it is protected from humoral factors. A multifocal to diffuse granulomatous lesion develops in the intestine and regional lymph nodes. Terminally a bacteremia and anergy develops. Bacteremia can also develop in infected animals by desensitization with johnin. The organism has been recovered from testes, semen, bulbourethral gland, prostate, seminal vesicles, mammary gland, uterus, and the fetus. Because
of these observations, congenital infections have been postulated. However, in a study by Merkal et al.,\textsuperscript{65} 75% of infected animals within a herd came from noninfected dams. During anergy, the organism may invade the cotyledons and result in abortion.\textsuperscript{66} Transuterine infection with subsequent development of paratuberculosis has not been documented.\textsuperscript{44} As many as 7% of infected animals shed the organism in the milk.\textsuperscript{29,58,59} This may also serve as a significant source of infection in young animals in addition to contaminated feces which may contain greater the $10^8$ organisms per gram.

Paratuberculosis is a slow spreading disease requiring an extended period of time for the development of clinical signs. Herds consequently become infected before the disease is recognized in individual animals. It has been shown that by the time the first infected animal is recognized, 38%-42% of the herd is infected.\textsuperscript{67-70} Annual death losses within a herd may be as high as 3%-10%. Clinical disease is usually associated with adult (>2 years) animals, however clinical disease has been seen in animals as young as 4 months of age.\textsuperscript{71} Parturition, a low plane of nutrition, high milk production, parasitic infection, grazing on mineral-deficient low lying wetlands, and various other stress factors have been incriminated in precipitating clinical disease.\textsuperscript{72-75}
Clinical Signs

Clinical signs usually begin as a nonresponsive, chronic or intermittent diarrhea. Periods of apparent remission occur which may last for weeks or months. Diarrhea subsides and weight gains occur during pregnancy, however at parturition diarrhea resumes, often more severe than previously. There may be intermittent fever, however appetite is usually good. During the terminal stages of the disease the diarrhea may become bloody, there is loss of appetite, emaciation, ventral edema, and death.

It has long been known that clinical signs and the severity of the lesions rarely correspond. In cattle the lesion is characterized as a diffuse granulomatous change, without necrosis, hyperemia, or reactionary fibrosis. Primary lesions are confined to the intestinal tract and regional lymph nodes, but there may be effusion into body cavities, serous atrophy of fat, and subcutaneous edema in the dependent areas of the carcass. The intestine is usually thickened, corrugated and highly folded. The folds remain when the segments of gut are stretched. In animals infected with pigmented strains of *Mycobacterium paratuberculosis* the intestinal mucosa may take on a yellow-orange hue. Lesions may be found from the duodenum to rectum, but are
most common in the terminal ileum. In younger animals there may be no intestinal thickening, instead a hemorrhagic enteritis occurs. In regional mesenteric lymph nodes there is a granulomatous lymphadenitis with marked lymph node enlargement. Mesentric lymphatics may also be prominent. Lesions occasionally occur in other organs with the liver being the most common secondary organ affected.

Pathology

Histologically the affected intestine contains numerous foamy macrophages within the lamina propria and occasionally in the submucosa. Early lesions appear tuberculoid (nodular) and rapidly coalesce to become lepromatous (diffuse). Langhans' type giant cells may or may not be a prominent feature. Acid-fast stains reveal the presence of numerous bacilli within the macrophages and giant cells. Occasionally, the bacilli are sparse or may not be evident at all. The earliest lesions seen in experimentally infected calves are focal aggregates of macrophages in Peyer's patches and in the villus tips. Mast cell mediated enteropathy of the cranial portions of the intestinal tract is likely to be another feature in the early stages of paratuberculosis.

In regional lymph nodes there are multifocal
accumulations of macrophages and giant cells primarily within the cortex. These nodular accumulations frequently coalesce. Acid-fast bacilli are usually demonstrable in these aggregates as well. In the liver there may be multifocal granulomas and/or pericholangitis. Acid-fast bacilli are usually not observed in hepatic lesions.\textsuperscript{76,84}

Intimal fibrosis and mineralization occur in the abdominal aorta and heart\textsuperscript{38,87,89}. A fibrinoid or amyloid-like substance may be present in small vessels of lymph nodes, adrenals, udder, and renal glomeruli.\textsuperscript{44} Neurologic lesions such as degenerative changes in the sciatic nerves and brachial plexus resembling lepromatous leprosy have been reported.\textsuperscript{89} Acid-fast bacilli are also present in these lesions.

**Diagnosis**

The antemortem diagnosis of paratuberculosis is hindered by the absence of sensitive tests. It should be kept in mind that immunologic methods give an indication of exposure and not infection and to distinguish between resistant and infected animals may be impossible using immunologic methods. Numerous diagnostic methods have been employed. These include intradermal and intravenous johnin tests, complement fixation, hemagglutination, agar gel immunodiffusion, enzyme-linked
immunosorbent assay (ELISA), radioimmunoassay, fluorescent antibody, lymphocyte transformation, migration inhibition, leukocyte migration, immunoperoxidase, mucosal and lymph node biopsy, and fecal culture. None of these tests have proven to be completely accurate, providing variable percentages of false negatives and false positives. The available number of confirmed ELISA test results is too small to establish the accuracy of the test, but initial results are promising. It has been speculated that false positives only occur as the result of cross-reactivity with other mycobacteria and that such spurious results would be eliminated if pure and species specific antigens are utilized. This does not appear to be true because some animals develop a protective immunologic response, eliminate the organism, and become positive by serologic testing. These animals are then resistant to reinfection but continue to be serologically positive and should therefore not be culled, but be retained as breeding stock.

False negative results occur due to anergy or to antigen masking factors. The failure to respond to antigen usually occurs at the terminal stages of the disease, but anergy may occur at any time during chronic infection. In general, cell mediated immune responses are susceptible to tolerance. An animal which is
immunologically tolerant may react humorally but fails to develop an adequate cell mediated response. An initial serologically positive response with a subsequently negative cellular response may be interpreted as anergy in the infected state or following recovery from a previous infection.

Serologic testing on an individual animal basis is of little value. Most infected herds, however, have a prevalence of infection of 36%-42%. Therefore, testing 10 out of 100 animals in an infected herd will result in a 98% probability for finding a positive response.

Although probably the most variable in its results and interpretation, intradermal johnin testing provides an easy and convenient method of diagnosis which can be used on the farm. The test is performed similar to tuberculin testing, except that results are read in 24-48 hours in cattle. A change in skin thickness greater than 5 mm is considered a positive test. Repeated tests can be performed at any time, but different sites must be used; apparently a local desensitization occurs at the site of injection.

Intravenous johnin tests provide greater accuracy than intradermal testing, but requires 2-4 ml of antigen. A positive reaction is indicated by a febrile response or a change in the neutrophil:lymphocyte ratio. A rise in rectal temperature of 1.5°C within
six hours post-injection is considered a positive response. A shift in the neutrophil:lymphocyte ratio greater than 2:1 at six hours post-injection is also considered a positive test result.

Microbiology

The only means by which a definitive etiologic diagnosis can be made is by cultivation of the organism either from feces or infected tissues. The organism is usually grown on Herrold's egg yolk media and identification is based on mycobactin dependence. The suspected material should be inoculated on two slants, one with and one without mycobactin. Mycobactin P and mycobactin J will both support growth, however culture times are reduced from 8 weeks to 4-6 weeks by using mycobactin J. Mycobactin J is derived from *Mycobacterium paratuberculosis* Ames Strain 18 and enhances growth of strains which could otherwise not be cultured. Fecal cultures further complicate the isolation problem because of the necessity of decontamination. The feces (and contaminated tissue) are suspended in benzalkonium chloride to destroy saprophytic mycobacteria, fungi, and other bacterial contaminants prior to inoculation onto appropriate media. Hexadecylpyridinium chloride at a concentration of 0.75% has also been used and may prove to be a more effective
decontaminant than benzalkonium chloride. Even with these decontaminating procedures, saprophytic overgrowth frequently occurs. Some isolates of *Mycobacterium paratuberculosis* are highly sensitive to the decontamination solutions.\textsuperscript{44} The minimum number of organisms shed in the feces which can be detected by fecal culture appears to be about 100 per gram of feces,\textsuperscript{106} however methods are available which can detect 1 organism/gram of feces.\textsuperscript{88}

**Vaccination and Treatment**

Vaccines have been used in attempts to control the disease. A number of bacterins, both attenuated and unattenuated have been evaluated,\textsuperscript{116-121} In addition, heat-killed organisms,\textsuperscript{122-124} and disrupted fragments of *Mycobacterium paratuberculosis*\textsuperscript{117,124,125} have been used. The effectiveness of these vaccines has been disputed. However, the consensus is that vaccination will reduce the number of clinical cases, decrease the number of animals excreting the organism, and decrease the number of animals with detectable intestinal lesions.\textsuperscript{121,124,157,158} Vaccination does not confer absolute immunity as some vaccinates may develop disease or shed the organism.\textsuperscript{118,120,122-127}

The main disadvantage to vaccination is that cattle
vaccinated with *Mycobacterium paratuberculosis*-origin bacterins develop hypersensitivity to johnin, avian PPD, tuberculin, and *M. bovis* PPD.\textsuperscript{127} The vaccine is usually administered at 1-35 days of age and immunity lasts about 18 months.\textsuperscript{125-126} Revaccination is not recommended because of the possibility of decreased resistance following revaccination.\textsuperscript{33,121,128}

The paratuberculosis organism is susceptible to numerous antituberculous drugs in vitro, however treatments have not been successful.\textsuperscript{107-115} In these attempts, animals frequently improve clinically, however continue to shed the organism and some animals eventually succumbed to the disease.

**Immunoreactive Substances of Mycobacteria**

The various species of mycobacteria have numerous similar chemical and adjuvant properties. All contain various mycosides, trehalose dimycolate, muramyl dipeptide, phosphotides, sulfatides, Waxes D, and water soluble adjuvants which make them unique from a immunological aspect. The most common factor in the mycobacterial component of Freund's complete adjuvant having immunoadjuvant activity is known to be N-acyl-muramyl-L-alanyl-D-isoglutamine, otherwise known as muramyl dipeptide or MDP.\textsuperscript{129} The multiplicity of MDP
effects have been outlined by Masek. A pyrogenicity effect of MDP was noted soon after its discovery. Somnogenicity, antinociceptivity, immunoadjuvanticity, anti-inflammatory, and hepatoprotective effects have been also described. The target cell of MDP appears to be the macrophage. Lymphocyte proliferative responses seem to be secondary to the need for macrophages for T-cell activation.

Another important adjuvant of mycobacterial origin is trehalose dimycolate or cord factor. The name "cord factor" came from the observation that M. tuberculosis in culture formed serpentine cords in parallel arrangements. Cord factor also has the ability to activate macrophages and cause secretion of mitogenic factors by mouse thymocytes in conjunction with addition of muramyl dipeptide. Cord factor is granulomagenic, probably by virtue of its chemotactic and stimulant properties for macrophages and has antitumor properties.

The mycosides are a diverse group of glycolipids which have become essential in serotyping organisms in the Mycobacterium avium-intercellulare-scrofulaceum complex. The mycosides of bovine origin are designated as mycoside B and are glycosylated phenolic phthicerol esters. Studies by Brennan and colleagues demonstrated that the glycosylated mycosides confer
antigenic and serologic specificity through an extremely small oligosaccharide. The same oligosaccharide has been identified from prototype strains of *Mycobacterium paratuberculosis* supplied by the National Disease Center in Ames, Iowa.\(^{144}\) The compound is also identical to a previously isolated oligosaccharide from a strain of *M. avium*\(^{213,214}\) and it has been suggested that *M. paratuberculosis* may in fact be a specific *M. avium* serotype.\(^{213}\) Additional studies by McFadden and others have shown that *M. avium* and *M. paratuberculosis* can be distinguished on the bases of differences in their restriction fragment length polymorphisms.\(^{215}\) Recently lipoarabinomannan and lipid free arabinomannans have been shown to be potent immunoreactive substances of mycobacteria and in particular *Mycobacterium paratuberculosis*, and these antigens have been used with considerable success in enzyme immunoassays for Johne's disease.\(^{218}\)

**Immunology of Paratuberculosis**

There is a paucity of information on the cellular immunology of Johne's disease compared to tuberculosis or leprosy. The clinical signs in cattle affected with Johne's disease have been postulated to result from an allergic response to products of the organism.\(^{146}\) It is
speculated that an antigen-antibody reaction in the infected tissue may cause the release of significant amounts of histamine resulting in diarrhea. The reaction of antigen with competent lymphocytes would then cause the release of nonspecific pyrogen and cytotoxins resulting in a febrile response, anemia, and emaciation. With these speculations in mind, Merkal and Witzel used whole blood, white blood cells, plasma, and febrile plasma to passively transfer johnin hypersensitivity to noninfected calves. All blood elements except for non-febrile plasma were able to confer johnin hypersensitivity with temporary fever and diarrhea.

Plasma factors which suppress in vitro lymphocyte transformation have been identified in lepromatous leprosy and paratuberculosis. In addition, in lepromatous leprosy there is an extensive replacement of paracortical areas in regional lymph nodes by histiocytes. A similar replacement also occurs in Johne's disease. Therefore, it appears that the destruction of paracortical areas in lymph nodes may result in the inability to mount an effective immune response.

Crohn's disease is a chronic inflammatory condition of the intestinal tract which affects humans. Histologically, the intestinal lesions are composed of a diffuse, intense accumulation of macrophages in the
lamina propria and submucosa, not unlike bovine paratuberculosis. For years, the cause of Crohn's disease has been unknown, however recently a mycobacterium has been isolated from several clinical cases.\textsuperscript{216} The mycobacterial agent has been identified as \textit{M. paratuberculosis},\textsuperscript{216,217} and through the use of DNA probes has been shown to be indistinguishable from the Johne's bacillus.\textsuperscript{215}

Cattle affected with Johne's disease should provide an excellent model for the study of the pathogenesis of chronic intracellular infections. Due to the similarities of the various mycobacterial organisms and the similarities in disease conditions, particularly lepromatous leprosy, Crohn's disease and Johne's disease, it can be postulated that there may also be similarities in the cellular immunology.
Cellular Immunologic Events in Mycobacterial Infections

Although cellular immunity has only been superficially examined in Johne's disease, it has been closely examined in other mycobacterial diseases. It remains to be shown whether many of the observations noted in other mycobacterial diseases may also apply to *Mycobacterium paratuberculosis* infections.

Initial cellular events following the introduction of mycobacterial antigen involve presentation of antigen to appropriate lymphocytes. Accessory cells such as resident macrophages, dendritic cells, Langerhans cells and M cells provide this function. Resident macrophages may not be as important as other non-lymphoid accessory cells.\textsuperscript{150-152} If major histocompatibility class (MHC) II antigens (Ia antigen) are expressed on both the accessory cells and T-lymphocytes, the foreign antigen can be adequately presented and invoke the release of soluble mediators such as interleukin 2 (IL-2) and gamma interferon. IL-2 is secreted by T-cells of the helper cell phenotype.\textsuperscript{153,154} Normally IL-2 secretion can be invoked by various plant lectins such as phytohemagglutinin, conconavalin A, or pokeweed mitogen and numerous antigens. Calcium ionophore A 23187 in the presence of phorbol myristate acetate has also recently been shown to be a potent inducer of IL-2 synthesis.\textsuperscript{155} The IL-2 invokes increased IL-2 receptor expression and
proliferation of a suitable clone of T-lymphocytes (helpers, cytotoxic, and natural killers).\textsuperscript{156,157} The IL-2 also regulates and augments interferon production\textsuperscript{158} which in turn is necessary for increased Ia antigen expression.\textsuperscript{159,160} Without Ia antigen expression, accessory cell and lymphocyte interaction do not occur. In certain mycobacterial infections there is a decrease in macrophage Ia expression.\textsuperscript{163} Inhibition of IL-2 by muramyl dipeptide, a constituent of mycobacterial organisms, has been demonstrated.\textsuperscript{161} A decrease in IL-2 production may explain the paucity of lymphocytes in histologic lesions of chronic Johne's disease. Mycobacterium infected macrophages have also been shown to have decreased Fc receptor expression.\textsuperscript{165}

It has been shown that lymphocytes incubated in vitro with mycobacterial antigens produce a population of lymphocytes that suppresses IL-2 production in normal lymphocytes. These antigens do not affect IL-1 production by normal monocytes.\textsuperscript{162}

**Macrophage Function**

The role and function of the macrophage in mycobacterial infections is controversial. Various research groups have obtained conflicting results. Much of this conflict may be due to the fact that most studies are performed on blood-derived macrophages or monocytes
instead of those mononuclear phagocytes actively participating in the local lesions.

Macrophages infected with mycobacteria have a decreased ability to kill other bacteria and phagocytize zymosan.\textsuperscript{164,165,226} In addition, it has been shown that phagosome-lysosome fusion is inhibited in mycobacterium-infected macrophages when intact organisms such as \textit{Bacillus subtilis} and \textit{Mycobacterium avium} are presented to the affected macrophage.\textsuperscript{166}

There may be a decrease in oxidant metabolite production. In addition to being cytocidal to microorganisms, the oxidants produced by phagocytic cells are necessary for production of carbonyl radicals on the surface of lymphocytes which is a necessary prerequisite for the production of IL-2.\textsuperscript{167} Infected macrophages are also known to inhibit lymphocyte blastogenesis and lymphokine production.\textsuperscript{168} This activation may be inhibited by calcium channel blockers and calmodulin antagonists (i.e. acepromazine).\textsuperscript{169}

Macrophages are responsible for IL-1 production which in turn stimulates T-cells to express receptors and secrete IL-2.\textsuperscript{170-173} In patients with lepromatous leprosy there is an increase in prostaglandin-E\textsubscript{2} which is a potent inhibitor of IL-1 production\textsuperscript{219}. This can be demonstrated in vitro with \textit{M. leprae} stimulated macrophages from lepromatous individuals.\textsuperscript{174}
Drutz et al has shown that leukocytes (monocytes, macrophages, and polymorphonuclear cells) from lepromatous and tuberculoid leprosy patients possess equivalent microbiocidal activity towards *Listeria monocytogenes*, *E. coli*, *Proteus vulgaris*, *S. aureus*, and *Candida albicans*. In addition, Ridel has shown that there is no difference in IL-1 production in lepromatous patients compared to healthy individuals. In Crohn's disease there appears to be an increase in IL-1 production when induced and as a spontaneous phenomenon. This implies a state of chronic activation or an inherent macrophage defect.

It has been documented that gamma-interferon is an important activator of macrophages. Nogueira demonstrated that peripheral blood lymphocytes of leprosy patients fail to produce interferon and that production was restored with the addition of purified IL-2.

Kaplan reported that macrophages from lepromatous leprosy patients were deficient in hydrogen peroxide production, but responded similarly to those from healthy individuals after the addition of interferon and that this effect was independent of prior macrophage ingestion of *M. leprae* bacilli. This study concluded that the immune defect was a lack of response to *M. leprae* by the patients' T-cells rather than a defective response of macrophages to interferon.
It has been shown in patients with lepromatous leprosy that there was no increase in C-reactive protein\textsuperscript{182}. It is known that IL-1 is responsible for C-reactive protein production in acute phase inflammatory responses.\textsuperscript{183,184} Together these findings suggested that IL-1 production may also be decreased in mycobacterial infections.

Macrophage lysates from lepromatous leprosy patients have been shown to produce non-dialyzable antigen specific suppressor factors.\textsuperscript{185,186} Proliferation of T-lymphocytes and macrophages promoted by conconavalin A and other mycobacteria were inhibited by these lysates.

Natural killer cells were essential in tumor surveillance and and destruction of parasitized cells (macrophages).\textsuperscript{187,188} Cytotoxic T-cells performed roughly the same function, however natural killer cells did not require MHC matching with target cells. In some mycobacterial infections, IL-2, which is important in killer cell proliferation, has been demonstrated to be deficient.\textsuperscript{189}

In patients with lepromatous leprosy a decrease in the T4/T8 ratio in the lesions indicated a relative decrease in helper cell activity, therefore the cells necessary for IL-2 production were deficient in number.\textsuperscript{190} In addition, studies using anti-Tac antibody labeling have shown that there was a decrease in IL-2
receptor expression, but lymphocyte function could not be fully restored with the addition of exogenous IL-2 alone\textsuperscript{191}.

**Immunomodulation**

Dialyzable leukocyte extracts containing transfer factor augmented cellular and humoral immunity.\textsuperscript{192} Transfer factor has been considered a portion of or the entire cell receptor for IL-2. Leprosy patients or paratuberculosis infected animals should benefit greatly from treatment with transfer factor. Supplementation with IL-2 may increase the immune response by reinstituting cellular proliferation of T-helper cells in those mycobacterial diseases in which IL-2 is deficient. Pharmacologic enhancement of macrophage function may lead to enhanced IL-1 production. This in turn may result in increased IL-2 receptor expression on lymphocytes\textsuperscript{193} and augmented proliferative and functional responses.

Since the discovery of transfer factor by Lawerence,\textsuperscript{194,195} viable leukocytes, leukocyte extracts, or dialysates of leukocyte extracts have been used to transfer delayed hypersensitivity to previously naive individuals. Clinical syndromes treated with transfer factor have included cancer immunotherapy,\textsuperscript{196-198} internal parasitism,\textsuperscript{199} lupus erythematosus,\textsuperscript{200}
sarcoidosis, vaccinia, coccidiomycosis, tuberculosis, lepromatous leprosy and numerous others. The results have been variable, but were often favorable.

Leukocyte lysates which contained inducer factors (transfer factor) and suppressor factors have been obtained from T-helper and T-suppressor cells, respectively. Much of the variability noted in clinical applications probably has been due to the fact that the crude lysates contained mixtures of both factors. The suppressor factor has also been used in the modulation of diseases such as lupus erythematosis and autoimmune thrombocytopenic purpura that are characterized by an undesirable immune response. Certain chemical and biological properties of transfer factor have been well documented. In dialysates, the active component was present in the 3.5 to 12kD molecular weight range. Transfer factor binds to specific antigen and anti-Ia antibody. The product was absorbed by T-suppressor cells and macrophages. In addition, transfer factor equipped nonimmune cells with an antigen-binding moiety. It has been postulated that transfer factor may be a dialyzable fragment of a T-helper cell receptor (possibly IL-2 receptor). The duration of effect of treatment with transfer factor has not been well
documented in every case. In a study using transfer factor as a preventative measure against varicella-zoster exposure, it was found that titers of treated, exposed patients who were protected from disease developed antibody titers that remained as high as those in infected individuals for 17 months. The transfer factor treated group also had an active cell mediated immunity as demonstrated by in vitro and skin tests for the same period of time.

In a study using transfer factor in lepromatous leprosy, donors for transfer factor were selected on the basis of positive skin reactivity and in vitro lymphocyte responses. Recipients were initially negative to skin tests using lepromin. After administration of transfer factor the recipients became skin test positive after the first or third days. In vitro testing of lymphocyte function (blastogenesis) was not altered. The recipients experienced erythema of the cutaneous lesions and occasionally fever. There was no alteration in histologic characteristics of the lesions when compared to pre-transfer factor lesions. The majority of the recipients reverted to a skin test negative status about 25 months after transfer factor administration. These changes, although shortlived were extremely promising when it is considered that only one injection of transfer factor was given. A multiple
dose trial appears indicated.

Chemotherapy

As previously stated, several anti-tuberculous drugs have been used to treat Johne's disease with little or no success. Clofazimine and isoniazid have been used most extensively although in a limited number of animals. A relatively new compound, Rifabutin (ansamycin LM 427), appeared promising. Preliminary results with rifabutin therapy in stump-tailed Macaques infected with M. paratuberculosis demonstrated remission of clinical signs and an extended life expectancy.

Objectives

The exact nature of the immune defect in mycobacterial infections has not been determined. Lymphocyte-macrophage interactions are extremely complex and difficult to dissect from one another. Transfer factor appears to augment lymphocyte function and not affect other aspects of cellular immunity. There are pharmacologic agents and other chemicals which augment monocyte/macrophage function with little or no effect on lymphocyte function. Proper macrophage function should also be restored if the infectious agent or antigen can be removed by an effective antimicrobial agent.
The objectives of this study were:

1. To determine the existence and nature of the cellular immune defect in cattle naturally infected with Mycobacterium paratuberculosis. Specific objectives included:
   
   (a) Skin hypersensitivity to johnin, M. bovis PPD, and keyhole limpet hemocyanin antigens.

   (b) Lymphocyte blastogenic responses, macrophage migration inhibition factor production, interleukin-1 release and interleukin-2 production in response to the skin test antigens.

2. To determine the response of cattle with chronic paratuberculosis and non-infected cattle inoculated with Mycobacterium paratuberculosis bacterin, live M. bovis BCG, and keyhole limpet hemocyanin in Freund's incomplete adjuvant to the specific parameters outlined in objective 1.

3. To determine the cell-mediated immune response in three naturally infected cattle with paratuberculosis using the assays outlined in objective 1 following weekly treatment for three weeks with specific transfer factor prepared to keyhole limpet hemocyanin and Mycobacterium paratuberculosis.
4. To determine the cell-mediated immune response in three naturally infected cattle with paratuberculosis using the assays outlined in objective 1 after daily treatment with 1200 mg rifabutin for three weeks.

5. To determine the extent of lesion alterations in ileal and ileocecal lymph nodes in the cattle described in objectives 3 and 4 before and after transfer factor and rifabutin treatment.
LYMPHOCYTE BLASTOGENESIS AND MACROPHAGE MIGRATION INHIBITION IN CATTLE WITH CHRONIC BOVINE PARATUBERCULOSIS BEFORE AND AFTER SENSITIZATION WITH M. PARATUBERCULOSIS, M. BOVIS AND KEYHOLE LIMPET HEMOCYANIN

Abstract
Cutaneous hypersensitivity, lymphocyte blastogenesis and macrophage migration inhibition in response to M. bovis PPD, johnin, keyhole limpet hemocyanin, and phytohemaglutinin were examined in 3 cattle naturally infected with Mycobacterium paratuberculosis and 3 non-infected cattle. Infected cattle had variable response to the skin test antigens and largest blastogenic responses to M. bovis PPD also with positive responses to johnin. No significant blastogenic responses were seen with keyhole limpet hemocyanin in either group. Blastogenic responses with phytohemaglutinin were similar in both groups. The results of macrophage migration inhibition paralleled the lymphocyte blastogenic responses to the antigens and mitogen. Sensitization
with *M. bovis* BCG, *M. paratuberculosis*, and keyhole limpet hemocyanin in Freund's incomplete adjuvant resulted in positive skin tests, blastogenic and migration inhibition responses in control cattle with *M. bovis* PPD, johnin, and keyhole limpet hemocyanin and no change in the phytohemaglutinin responses. There was no difference in pre- and post sensitization blastogenesis and inhibition of migration data in infected cattle. There was, however, a significant decrease in johnin and *M. bovis* PPD skin test reactivity and a failure to produce positive responses to keyhole limpet hemocyanin and *M. bovis* PPD after sensitization.

**Introduction**

Bovine paratuberculosis is a chronic enteric infection caused by *Mycobacterium paratuberculosis* which results in diarrhea, weight loss and eventually death. numerous serologic tests for the antemortem diagnosis of paratuberculosis have been used with inconsistent results. Lymphocyte blastogenesis (LB) and macrophage migration inhibition factor (MIF) assays have been suggested as good in vitro indicators of the cell mediated immune response. These assays have also been used for the diagnosis of paratuberculosis.
Anergy is known to occur in bovine paratuberculosis. Whether this anergy is complete or specific for only *M. paratuberculosis* has not been well characterized. In this study, cell mediated immune responsiveness was measured by skin testing and LB and MIF assays in cattle with chronic paratuberculosis before and after administration of sensitizing doses of *M. bovis* BCG, *M. paratuberculosis*, and keyhole limpet hemocyanin (KLH). This was done to determine whether or not cattle infected with *M. paratuberculosis* can distinguish between and mount a positive immune response to homologous and heterologous antigens as assessed by LB, MIF, and skin testing.

**Materials and Methods**

**Animals:**

Three adult Brahman-cross cattle diagnosed positive for bovine paratuberculosis by rectal biopsy, ileal biopsy and by enzyme-linked immunosorbent assay were donated to the Louisiana State University School of Veterinary Medicine by local cattle producers. These animals had a history of weight loss and intermittent diarrhea of at least one year. duration. In addition, 3 healthy control cattle were selected at random from the School of Veterinary Medicine teaching herd to serve as controls. Fecal cultures from control animals using Herrold's egg-yolk media with and without 2 μg/ml
mycobactin J (Allied Labs, Ames, IA) were negative after 6 months of incubation.

**Media and Reagents**

The culture medium used for the LB assays consisted of RPMI 1640 (GIBCO Laboratories, Chargin Falls, OH) supplemented with 4 mM L-glutamine and 10% fetal calf serum (FCS) without antibiotics (complete culture medium, CCM). Dulbecco's MEM (GIBCO Laboratories, Chargin Falls, OH) was used in the MIF assay. Phosphate-buffered saline (PBS) was used as a washing solution. Phytohemaglutinin (PHA), keyhole limpet hemocyanin (KLH) (Sigma Chemical Co., St. Louis, MO), johnin and *Mycobacterium bovis* (M. bovis) PPD (Veterinary Services Laboratory, Ames, IA) were diluted to appropriate concentrations (as determined by previous dose response trials) in CCM. *Mycobacterium bovis* (Trudeau Institute, Saranac Lake, NY), *Mycobacterium paratuberculosis* bacterin (Fromm Laboratories, Grafton, WI), and KLH in Freund's incomplete adjuvant (Sigma Chemical Co. St. Louis, MO) were used to sensitize both groups. Freshly prepared 

\[^{3}H\]thymidine in CCM was used to assess proliferative responses in blastogenesis assays.

**Skin Testing**

The cervical area of each cow was clipped and double
skin thickness measured prior to injection. Intradermal injections of 0.2 ml containing 1000 µg johnin, *M. bovis* PPD and KLH in PBS were given to each cow. For a negative control PBS was used. The change in double skin thickness was recorded after 48 hrs. The cattle were retested using the contralateral cervical area after sensitization. A change in skin thickness >5mm was considered a positive reaction.

**Blood Mononuclear Cells**

Heparinized whole blood was collected by venapuncture and diluted 1:1 with PBS. An equal volume of Histopaque-1.083 (Sigma Chemical Co., St. Louis, MO) was slowly injected underneath the blood using a 6 in, 18 ga needle. The blood-Histopaque gradient was centrifuged at 1000 X g for 45 min and the mononuclear cell band at the interface was removed and the cells washed three times with PBS. Platelet contamination was minimized by differential low-speed centrifugation during washing (250 X g for 10 min). Viability by trypan blue exclusion was >95%.

**Lymphocyte Blastogenesis**

Gradient-separated mononuclear cells were suspended in CCM at a concentration of 2 X 10⁶/ml and 100 µl aliquots were placed in the wells of a 96 well microtiter plate. To triplicate wells was added 100 µl
of one of the following in CCM: 50 μg/ml johnin, 50 μg/ml *M. bovis* PPD, 30 μg/ml KLH, 25 μg/ml PHA, and CCM without additive. The cells were incubated for 72 hrs., pulsed with 0.5 μCi/50μl [3H]thymidine, and collected 18 hrs later onto glass fiber disks using a semi-automated cell harvester (Skantron Inc., Sterling, VA). The disks were dried overnight at 60°C and placed in scintillation vials to which was added 3 ml of scintillation cocktail (Fluoralloy™ in toluene, Beckman Instruments, Fullerton, CA). The vials were placed in the dark overnight to minimize inherent chemiluminescence. Counts were made for 2 min on a Packard Tri-Carb 4640 beta counter (Packard Instrument Co., Downers Grove, IL). The data was expressed as the antigen cpm/media cpm stimulation index (SI). A SI ≥ 2.0 was assigned as a positive response as previously determined.  

**Migration Inhibition Factor Assay**

A microagarose droplet assay as described by Harrington and Stastny was used with minor modifications.  

Cell pellets containing 10⁸ gradient-separated mononuclear cells were combined with 100 μl of 2 X Dulbecco's MEM with 20% FCS and 100 μl melted agarose (SeaPlaque™, FMC Corporation, Rockland, ME). The cells, media and agarose were gently mixed and 1 μl droplets were placed in the center of the wells of a 96 flatbottom
well microtiter plate. The plate was refrigerated (4°C) for 10 min prior to the addition of antigens. To triplicate wells 100 μl CCM was added containing one of the following: 50 μg/ml johnin, 50 μg/ml M. bovis PPD, 50 μg/ml KLH, 25 μg/ml PHA and CCM without additive. After 48 hrs incubation each well was photographed and a 5 X 7 was print made. The two most symmetrical droplets from each treatment were selected and the distance from the edge of the droplet to the edge of the cell migration zone was measured. Three random measurements were made from each of the selected droplets and the mean and standard error was calculated. Data were expressed as % inhibition of migration: 
\[
\left(\frac{\text{mean migration distance of media control}}{\text{mean migration distance of antigen}}\right) - \text{mean migration distance of media control} \right) \times 100.
\]
A 20% inhibition was considered a positive MIF assay reaction.

Sensitization

Infected and non-infected cattle were inoculated subcutaneously with 8.0 mg KLH in Freund's incomplete adjuvant, 1.0 ml heat-killed M. paratuberculosis bacterin (twice the recommended dose), and 6 X 10^8 M. bovis BCG. All assays were repeated 3 weeks after the sensitizing injections.
Statistical

Differences in stimulation indices and % inhibition of migration in both the infected and non-infected groups were tested using Student's t-test. A significant difference was considered to be $P<0.05$.

Results

Before sensitization two of three infected cattle had a positive skin test reaction to johnin (table 1). Slight increases were seen with *M. bovis* PPD before sensitization and no significant changes were seen with KLH or the CCM control. No positive skin test reactions were seen in the non-infected group before sensitization (table 2). After sensitization the non-infected cattle had positive skin test responses to *M. bovis* PPD, johnin, and KLH (table 2) while infected cattle had no positive skin test reactions to any of the antigens used (table 1).

The LB assay was positive in all the infected animals before sensitization with johnin and *M. bovis* PPD (table 3). No positive responses were seen with KLH in either group. No significant ($P>0.05$) difference in PHA induced proliferation was seen in either group. After sensitization infected cattle showed no change whereas control cattle significantly ($P<0.05$) increased LB responses to all the sensitizing antigens (table 4). No
difference \( (P>0.05) \) in the PHA response was detected.

The results of the MIF (tables 5 and 6) assay paralleled the LB assay. Infected cattle had positive responses with \textit{M. bovis} PPD and johnin before sensitization with no change after sensitization. Control cattle significantly \( (P<0.05) \) increased responses to the sensitizing antigens. Stimulation with PHA consistently produced a \% inhibition of migration \( >98\% \) in both groups of cattle.

**Discussion**

The LB and MIF assays used in this experiment were accurate indicators of chronic paratuberculosis. Skin testing with johnin and \textit{M. bovis} PPD were not reliable as previously indicated.\(^8\) After administration of sensitizing doses of \textit{M. bovis} BCG, \textit{M. paratuberculosis}, and KLH the control cattle developed positive skin tests, LB, and MIF assay reactions which were significantly different than pre-sensitization results. There was no change after sensitization in LB and MIF assays in the infected group, however there was a significant decrease in johnin skin test reactivity. It has been been shown that infected cattle can be desensitized with administration of johnin.\(^8\) This may account for the decrease in skin reactivity with johnin, but does not account for the failure to develop a positive skin
reaction to KLH and M. bovis PPD after sensitization. The phenomenon of positive LB with concurrent negative skin test reactions as seen in this experiment has also been previously reported.10

The majority of previous studies using lymphocyte blastogenesis assays employed the whole blood technique.5,6,10,13 These studies revealed a significant number of false positives and negatives with marked individual variation. This has been attributed to serum factors in cattle with paratuberculosis which inhibit lymphocyte proliferation.9 Using a purified blood mononuclear cell assay improved the specificity of the technique markedly.2,16 In addition, heavily infected cattle have greater variation in LB than minimally infected cattle.10 Mycobacterial antigens other than johnin have been used in blastogenesis assays with varying results. In general Mycobacterium avium antigen provided equal or greater proliferative responses in infected animals6,13,16. It has been suggested that the LB assay can distinguish between M. paratuberculosis and M. bovis and that M. avium infections can be differentiated from M. bovis infections.2,16,17 The results of this experiment indicate that M. bovis PPD is a better test antigen in the detection of infected animals than johnin, and that this would make it impossible to distinguish between paratuberculosis and M.
bovis infections. The differences noted in these two mycobacterial antigens may be a reflection of different potencies of the preparations as the same absolute quantities were used.

In this experiment the LB and MIF assays have been demonstrated to be accurate immunologic tests for the antemortem diagnosis of bovine paratuberculosis. The unreliability of skin testing as a diagnostic tool has been further substantiated. Infected cattle fail to produce a positive cellular immune response to heterologous antigen at a dose shown to sensitize non-infected cattle.
Table 1. Delayed Cutaneous Hypersensitivity in *Mycobacterium paratuberculosis* Infected Cattle

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Cattle</th>
<th>Pre-Sensitization Change in Skin Thickness (mm)</th>
<th>Post-Sensitization Change in Skin Thickness (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cattle</td>
<td>#1</td>
<td>#2</td>
</tr>
<tr>
<td>PBS&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.0</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>PPD&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.3</td>
<td>3.8</td>
<td>3.6</td>
</tr>
<tr>
<td>johnin</td>
<td>3.5</td>
<td>11.1</td>
<td>9.8</td>
</tr>
<tr>
<td>KLH&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.0</td>
<td>0.4</td>
<td>0.2</td>
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</table>

<table>
<thead>
<tr>
<th>mean+SD</th>
<th>mean+SD</th>
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<tbody>
<tr>
<td>PBS</td>
<td>0.07±0.06</td>
</tr>
<tr>
<td>PPD</td>
<td>3.23±0.81</td>
</tr>
<tr>
<td>johnin</td>
<td>8.13±4.06&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>KLH</td>
<td>0.20±0.20</td>
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</tbody>
</table>

<sup>a</sup>Phosphate buffered saline.
<sup>b</sup>Derived from *M. bovis*.
<sup>c</sup>Keyhole limpet hemocyanin.
<sup>*</sup>Positive reaction (>5mm increase).
Table 2. Delayed Cutaneous Hypersensitivity in Healthy Control Cattle

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Cattle</th>
<th>Pre-Sensitization</th>
<th>Change in Skin Thickness (mm)</th>
<th>Post-Sensitization</th>
<th>Change in Skin Thickness (mm)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>#1</td>
<td>#2</td>
<td>#3</td>
<td>#1</td>
<td>#2</td>
</tr>
<tr>
<td>PBS\textsuperscript{a}</td>
<td>0.2</td>
<td>0.1</td>
<td>0.0</td>
<td>0.4</td>
<td>0.1</td>
</tr>
<tr>
<td>PPD\textsuperscript{b}</td>
<td>1.6</td>
<td>0.9</td>
<td>0.4</td>
<td>18.5</td>
<td>27.5</td>
</tr>
<tr>
<td>johnin</td>
<td>1.7</td>
<td>2.0</td>
<td>0.9</td>
<td>11.9</td>
<td>15.2</td>
</tr>
<tr>
<td>KLH\textsuperscript{c}</td>
<td>0.0</td>
<td>0.2</td>
<td>0.3</td>
<td>6.3</td>
<td>9.7</td>
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<table>
<thead>
<tr>
<th>Antigen</th>
<th>mean+SD</th>
<th>mean+SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>0.10±0.10</td>
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</tr>
<tr>
<td>PPD</td>
<td>0.97±0.60</td>
<td>23.07±4.50\textsuperscript{*}</td>
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<tr>
<td>johnin</td>
<td>1.53±0.56</td>
<td>13.86±1.74\textsuperscript{*}</td>
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<tr>
<td>KLH</td>
<td>0.17±0.15</td>
<td>9.13±2.60\textsuperscript{*}</td>
</tr>
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</table>

\textsuperscript{a}Phosphate buffered saline.
\textsuperscript{b}Derived from \textit{M. bovis}.
\textsuperscript{c}Keyhole limpet hemocyanin.

\textsuperscript{*}Significantly different than pre-sensitization (P<0.05).
Table 3. Lymphocyte Blastogenesis in *Mycobacterium paratuberculosis* Infected Cattle

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Pre-sensitization</th>
<th>Post-sensitization</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cpm</td>
<td>SI&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>media</td>
<td>773</td>
<td>1.0</td>
</tr>
<tr>
<td>PPD&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8,039</td>
<td>10.1</td>
</tr>
<tr>
<td>johnin</td>
<td>5,101</td>
<td>7.0</td>
</tr>
<tr>
<td>KLH&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1,391</td>
<td>1.5</td>
</tr>
<tr>
<td>PHA&lt;sup&gt;d&lt;/sup&gt;</td>
<td>17,624</td>
<td>23.8</td>
</tr>
</tbody>
</table>

<sup>a</sup>The mean SI was calculated by dividing the mean antigen cpm of triplicate assays from each of the 3 infected animals by the cpm of the media control. SD did not exceed 10% of the mean.

<sup>b</sup>Derived from *M. bovis*; <sup>c</sup>keyhole limpet hemocyanin; <sup>d</sup>phytohemaglutinin.

All antigen and mitogen responses are significantly different than the media control (*P*<0.05). No differences are apparent between pre- and post-sensitization data.
Table 4. Lymphocyte Blastogenesis in Healthy Control Cattle

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Pre-sensitization</th>
<th>Post-sensitization</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cpm</td>
<td>SI(^a)</td>
</tr>
<tr>
<td>media</td>
<td>1,113</td>
<td>1.0</td>
</tr>
<tr>
<td>PPD(^b)</td>
<td>1,001</td>
<td>0.9</td>
</tr>
<tr>
<td>johnin</td>
<td>890</td>
<td>0.9</td>
</tr>
<tr>
<td>KLH(^c)</td>
<td>1,447</td>
<td>1.5</td>
</tr>
<tr>
<td>PHA(^d)</td>
<td>22,371</td>
<td>20.8*(^e)</td>
</tr>
</tbody>
</table>

\(^{a}\)The mean SI was calculated by dividing the mean antigen cpm of triplicate assays from each of the 3 infected animals by the cpm of the media control. SD did not exceed 10% of the mean.

\(^{b}\)Derived from M. bovis; \(^{c}\)keyhole limpet hemocyanin; \(^{d}\)phytohemaglutinin.

*Significantly different than pre-sensitization data (P<0.05).

\(^{e}\)Significantly different than media control (P<0.05).
Table 5. Mean Percent Inhibition of Migration in *Mycobacterium paratuberculosis* Infected Cattle

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Pre-Sensitization</th>
<th>Post-Sensitization</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPD&lt;sup&gt;b&lt;/sup&gt;</td>
<td>76.2± 8.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>72.4± 9.3</td>
</tr>
<tr>
<td>johnin</td>
<td>56.6± 6.6</td>
<td>59.1± 7.7</td>
</tr>
<tr>
<td>KLH&lt;sup&gt;c&lt;/sup&gt;</td>
<td>19.5± 1.2</td>
<td>15.3± 7.0</td>
</tr>
</tbody>
</table>

<sup>a</sup>Mean±SE  
<sup>b</sup>Derived from *M. bovis*;  
<sup>c</sup>keyhole limpet hemocyanin.  

% inhibition of migration with phyohemalutinin exceeded 98% in all assays.  
A positive response is >20% inhibition.  
No significant difference after sensitization.
Table 6. Mean Percent Inhibition of Migration of Healthy Control Cattle

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Pre-Sensitization</th>
<th>Post-Sensitization</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPD&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11.7± 3.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>37.0± 5.1&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>Johnin</td>
<td>8.0± 4.3</td>
<td>33.6± 4.0&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>KLH&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5.5± 2.8</td>
<td>23.8± 4.6&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>Mean±SE  
<sup>b</sup>Derived from *M. bovis*; <sup>c</sup>keyhole limpet hemocyanin.  
<sup>*</sup>Significantly different than pre-sensitization data (P<0.05).
LITERATURE CITED


CHAPTER 3

INTERLEUKIN-1 RELEASE BY BLOOD MONOCYTES FROM CATTLE NATURALLY INFECTED WITH MYCOBACTERIUM PARATUBERCULOSIS

Abstract

Interleukin-1 (IL-1) release from blood monocytes was measured using a mouse thymocyte proliferation assay. Levels were determined in 3 cattle naturally infected with Mycobacterium paratuberculosis and 3 non-infected cattle. Release of IL-1 was measured before and after sensitization with heat-killed Mycobacterium paratuberculosis, live Mycobacterium bovis BCG, and keyhole limpet hemocyanin. Monocytes from infected cattle before in vivo sensitization spontaneously released high levels of IL-1 and increased IL-1 release in response to bacterial lipopolysaccharide. Non-infected cattle had increased IL-1 release when blood monocytes were stimulated with M. bovis PPD, lipopolysaccharide, and johnin. Johnin produced the greatest response. Release of IL-1 after sensitization was unaltered in both groups. These data suggest either a chronic state of activation in blood monocytes...
or a defective regulatory mechanism for IL-1 in cattle infected with *Mycobacterium paratuberculosis*.

**Introduction**

Bovine paratuberculosis (Johne's disease) is a chronic enteric infection caused by *Mycobacterium paratuberculosis* most often affecting ruminants.\(^{11}\) The disease has a marked histopathologic resemblance to lepromatous leprosy and Crohn's disease and has been proposed as an animal model for Crohn's disease.\(^{28}\) Based on the similarity of the enteric lesions, it may be postulated that similar inflammatory and immunologic mechanisms occur in these diseases. Measurement of cytokine release and function in bovine paratuberculosis has been limited to the study of migration inhibition factor\(^{3}\) and indirect evaluation with lymphocyte blastogenic responses.\(^{6,13,14,19,21}\)

Interleukin-1 (IL-1) produced by antigenic stimulation of macrophages (M\(\Phi\)) has many molecular forms and functions.\(^{12}\) Its relationship to a variety of different disease conditions has also been examined. Spontaneous release of IL-1 from blood monocytes, M\(\Phi\) or alveolar M\(\Phi\) has been noted in patients with sarcoidosis,\(^{20}\) tuberculoid leprosy,\(^{35}\) scleroderma,\(^{1}\) tuberculosis,\(^{9}\) and Crohn's disease.\(^{30}\) In lepromatous leprosy blood monocyte-derived M\(\Phi\) fail to produce IL-1
initially, but can produce normal levels when cyclooxygenase inhibitors, such as indomethacin, are added to the IL-1 producing blood cells. This restoration effect was seen only in response to *Mycobacterium leprae*. In other disease conditions such as sarcoidosis, tuberculosis, and Crohn's disease, IL-1 was examined because of the central role of the MΦ in the lesions of these diseases. In scleroderma, the principle lesion component is an overabundance of collagen with the MΦ having only a minor role in lesion formation. However, examination of the role of IL-1 in this disease is also warranted because of its ability to induce fibroblast activation and proliferation.

The role of IL-1 in the pathogenesis of bovine paratuberculosis and an evaluation of host immune status has not been previously examined. As the MΦ is the main cellular component in the lesions of paratuberculosis, the ability of blood monocytes to produce and release IL-1 in cattle with chronic paratuberculosis was measured. Alterations in IL-1 release were also measured after sensitization with *Mycobacterium paratuberculosis* (M. paraTB), *Mycobacterium bovis* (M. bovis), and keyhole limpet hemocyanin (KLH).
Materials and Methods

Animals:

Three adult Brahman-cross cattle diagnosed positive for Johne's disease by rectal biopsy, ileal biopsy and by enzyme-linked immunosorbent assay as previously described.\[^{39}\] were donated to the Louisiana State University School of Veterinary Medicine by local cattle producers. These animals had a history of weight loss and intermittent diarrhea for at least one year. Non-infected animals were selected at random from the School of Veterinary Medicine teaching herd to serve as controls. Fecal cultures from control animals incubated on Herrold's egg-yolk media with and without 2 μg/ml mycobactin J (Allied Labs, Ames, IA) were negative after 6 months.

Media and Reagents:

Culture medium (RPMI 1640, GIBCO Laboratories, Chargin Falls, Ohio) supplemented with 4mM L-glutamine and 2% heat-inactivated fetal calf serum (FCS) without antibiotics was used for cell cultures from which IL-1 supernatants were prepared. Similar medium was used for the IL-1 assay with the addition of 10% FCS. Phosphate-buffered saline (PBS), pH = 7.4, was used as a washing
solution. Concanavalin A (Con A), KLH, *Serratia marcescens* LPS (Sigma Chemical Co., St. Louis, MO), johnin and *M. bovis* PPD (Veterinary Services Laboratory, USDA, Ames, IA), were diluted to the appropriate concentrations (as determined by previous dose response trials) in media with 2% FCS. Infected and non-infected cattle were inoculated subcutaneously with 8.0 mg KLH in Freund's incomplete adjuvant (Sigma Chemical Co., St. Louis, MO), 0.5 ml heat-killed *M. paraTB* in oil (Fromm Laboratories, Grafton, WI), and 6 X 10^8 *M. bovis* BCG (Trudeau Institute, Saranac Lake, NY). In vivo sensitization was evaluated by skin test reactivity, migration inhibition, and blastogenic responses using the sensitizing antigens (Chapter 2).

**Preparation of Monocytes:**

Blood (40 ml) was collected using 50 U/ml preservative-free heparin (Sigma Chemical Co., St. Louis, MO) as an anticoagulant. After diluting the blood 1:1 with PBS and equal volume of Histopaque^R^ (1.083 g/ml, Sigma Chemical, St. Louis, MO) was injected underneath the diluted blood using a 6 in, 18 ga needle. The blood-Histopaque^R^ gradient was centrifuged at 1000 X g for 45 min and the peripheral blood mononuclear cell layer at the interface was harvested. Cells were washed three times in PBS and suspended at a final concentration of 8
X $10^6$/ml in media with 2% FCS. Platelet contamination was minimized by differential low speed centrifugation during washing (250 X g for 10 min). The cells were incubated in a 75 cm$^2$ plastic culture flask (15 to 20 ml per flask) for 1 h. Non-adherent cells were removed by vigorously washing 5 times with PBS. Adherent cells were removed using a plastic cell scraper and suspended in media containing 2% FCS at a concentration of 1 X $10^6$/ml (trypan blue exclusion showed a viability of >90%). Recovered adherent cells consisted of >95% monocytes with slight platelet contamination. Aliquots of adherent cells (1ml) were placed in wells of a 24 well flat bottom plate (Corning Glass Works, Corning, NY).

Preparation of IL-1 Containing Supernatants:

To 1 ml of 1 X $10^6$ adherent cells/ml from each animal was added equal volumes of the following; media with 2% FCS, 50 µg/ml johnin, 50 µg/ml M. bovis PPD, 50 µg/ml KLH, and 20 µg/ml LPS. Cells were cultured for 24 hrs at 37°C and 5% CO$_2$. Supernatants were collected and maintained at -60°C until assayed (within 2 weeks).

IL-1 Assay:

The assay for IL-1 followed the guidelines as
reviewed by Gearing, et al\textsuperscript{15} with minor modifications. Adherent cell supernatants were thawed and serial two-fold dilutions were made with media to 1:64. The thymuses from 6 wk old C3H/HeJ mice (Jackson Laboratories, Bar Harbor, ME) were used in all assays. Each thymus was aseptically removed and teased apart in 5 ml of media. The thymocytes and thymic tissue were allowed to sediment at 1 X g for ten minutes. About 4 ml of cell suspension was withdrawn and adjusted to a final concentration of 5 X 10\textsuperscript{6}/ml in media containing 10% FCS and 1.5 \( \mu \)g/ml Con A. Viability assessed by trypan blue exclusion was >95%. 100 \( \mu \)l aliquots of thymocytes were added to the wells of a 96 flatbottom well culture plate. An equal volume of the varying dilutions of adherent cell supernatants was added to the thymocyte cultures. After incubation for 48 hrs, 0.5 \( \mu \)Ci/50\( \mu \)l \( ^{3} \text{H} \)thymidine in RPMI with 10% FCS was added to each well. Cells were harvested after 18 hr incubation onto glass fiber discs using a semi-automated cell harvestor (Skraton Inc., Sterling, VA). Glass fiber discs were dried overnight at 60\textdegree{}C, placed in scintillation vials and 3 ml of scintillation cocktail (Fluoralloy\textsuperscript{TM}, Beckman Instruments, Fullerton, CA.) was added. The samples were placed in the dark overnight to minimize inherent chemiluminescence before beta counting. Counts were made for two minutes on a Packard Tri-Carb 4640 beta counter
Statistical Analysis:

Wilcoxon's rank sum test was used to identify treatment group differences in IL-1 release; a difference was considered significant at P<0.05.

Results

The concentration of IL-2 in the monocyte-derived supernatants was negligible by the Con A blast assay. The results of the mouse thymocyte proliferation assay before sensitization are shown in Table 1. Supernatants obtained from media, KLH, M. bovis PPD, and LPS-stimulated monocytes induced thymocyte proliferative responses which were significantly (P<0.05) greater in the infected group than in noninfected controls. Proliferative responses produced by johnin stimulated monocyte supernatants were not significantly (P>0.05) lower in the infected group. The LPS-stimulated supernatants produced significantly (P<0.05) greater proliferative responses when compared to the media.
control in both groups. The control group monocyte response was significantly (P<0.05) greater with johnin antigen when compared to the other antigens, although all treatments are significant (P<0.05) compared to responses obtained with the media control. The thymocyte proliferative responses with M. bovis BCG, M. paraTB, and KLH in the same groups of animals after sensitization is presented in Table 2. Although the cpm differed greatly before and after sensitization, stimulation indices for the IL-1 responses were remarkably similar. There was a significant (P<0.05) decrease in cpm values corresponding to all stimuli in both groups of animals. Stimulation with johnin remained significantly (P<0.05) higher in the non-infected group after sensitization. The infected and non-infected groups had similar KLH and PPD responses.

Discussion

The lesions of bovine paratuberculosis typically reveal a marked infiltration of MΦ containing large numbers of M. paraTB organisms in the lamina propria and submucosa of the small intestine and in the cortex and medulla of mesenteric lymph nodes. Disseminated disease is rare, but has been reported4,11,18 and is felt to be the result of terminal anergy.11 As the MΦ is the main lesion component and provides a major initial defense mechanism against mycobacterial disease,
investigation in MΦ function with respect to cytokine production and/or release seems warranted.

It has been shown that bovine blood monocytes and monocyte derived MΦ readily phagocytose M. paraTB, but fail to kill the organism.5,37 In other mycobacterial diseases, such as Mycobacterium leprae infections, MΦ react similarly in that there is a failure to kill the organism or other phagocytosed organisms even when M. leprae-burdened macrophages are exposed to exogenous interferon gamma.32

In this study the blood monocytes from M. paraTB infected cattle spontaneously release IL-1. This phenomenon has been seen in other granulomatous diseases such as scleroderma,1 sarcoidosis,20 tuberculoid leprosy,35 and Crohn's disease.30 The similarities between bovine paratuberculosis and Crohn's disease extend beyond lesion morphology and spontaneous IL-1 release by blood monocytes. Recently a mycobacterial agent has been isolated from several Crohn's patients.10,33 Three separate isolates have been shown to be indistinguishable from M. paraTB ATCC 19698 by using DNA probes which distinguish between mycobacterial species.26 The spontaneous release of IL-1 by alveolar MΦ in sarcoidosis may be partially accounted for by the low levels of prostaglandin E₂ (PGE₂) produced by these cells in vitro.2 Prostaglandin-E₂ is a known
autoregulatory monokine of IL-1 production.\textsuperscript{15,24} In lepromatous leprosy the role of PGE\textsubscript{2} in IL-1 regulation has been further demonstrated by restoration of IL-1 release by addition of indomethacin to the assay system\textsuperscript{29}. Although not examined in this experiment, defective or depressed production of arachidonic acid metabolites may be responsible for high spontaneous IL-1 release from blood monocytes similar to that seen in alveolar MΦ in sarcoidosis.\textsuperscript{2}

The intense thymocyte proliferative responses obtained by supernatants from johnin stimulated monocytes in the control cattle may be attributed to certain immunoreactive substances of mycobacterial origin known to be potent inducers of IL-1. Muramyl dipeptide is a constituent of most if not all mycobacterial cell walls which has been shown to induce IL-1.\textsuperscript{16,34} Trehalose dimycolate alone or in conjunction with muramyl dipeptide has also been shown to increase production of a thymocyte mitogenic protein,\textsuperscript{34} which is probably analogous to IL-1. Increased (P<0.05) but lower response to \textit{M. bovis} PPD than johnin may represent differing amounts of these immunoreactive agents in these two organisms, although this has not been substantiated.

Keyhole limpet hemocyanin is known to possess dose dependent mitogenic properties for murine lymphocytes\textsuperscript{23}. This may account for the moderate responses seen in the
control group with KLH. A second explanation, still to be demonstrated, may be that KLH shares similar properties with some phorbol esters. Phorbol esters such as 10-O-tetradecanoyl-phorbol-13-acetate have been shown to induce lectin-driven T-cell proliferation independent of accessory cells and IL-1.7,10,17,22

Sensitization was confirmed in the control cattle by positive intradermal reactions, lymphocyte blastogenesis (LB), and migration inhibition factor (MIF) assay (chapter 2). The infected cattle showed no evidence of sensitization using these methods, although the same doses of sensitizing antigen were used in both treatment groups. Anergy, known to occur in bovine paratuberculosis,4,11 may provide an explanation for in vivo sensitization failure in infected cattle.

On initial consideration it may be expected that if a positive response to a sensitizing antigen is obtained in LT and MIF assays, then an increase in IL-1 release should also be expected, however this is probably not the case. In a study by Chatila et al8 it has been shown that the necessary requirement for resting T-cell proliferation is contact by the accessory cell or monocyte. Interleukin-1 alone could not promote mitogen driven proliferation of purified T-cell cultures. With suboptimal concentrations of monocytes (0.1%), exogenous IL-1 increased lymphocyte proliferative responses in a
dose dependent manner. Higher concentrations of mononocytes, similar to that seen in peripheral blood, produced lymphocyte proliferative responses independent of the concentration of exogenous IL-1.\textsuperscript{8} It was also shown that monocyte contact is essential for lymphocyte proliferation. This may be due to surface associated IL-1\textsuperscript{125} or possibly a currently unknown mechanism. In in vitro assay systems such as LT and MIF the necessary accessory cell-T-cell contact is established with optimal concentrations of monocytes and the results are not dependent on secreted IL-1.

The results indicate that there may be an abnormal regulatory mechanism for IL-1 secretion in bovine paratuberculosis. This may reflect abnormalities in arachidonic acid metabolism or some other cytokine yet to be identified as a modulator of IL-1. Alternatively spontaneous IL-1 release may be the result of chronic activation due to the large antigenic burdens in cattle infected with \textit{M. paraTB}. 
TABLE 1. Mean Mouse Thymocyte Proliferation By Monocyte Supernatants In The Presence Of Con A Before Sensitization

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Infected Group</th>
<th>Noninfected Group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cpm&lt;sup&gt;a&lt;/sup&gt;</td>
<td>SI&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>media</td>
<td>10,191</td>
<td>1.000</td>
</tr>
<tr>
<td>johnin</td>
<td>8,158</td>
<td>0.866</td>
</tr>
<tr>
<td>KLH</td>
<td>13,363</td>
<td>1.299</td>
</tr>
<tr>
<td>PPD</td>
<td>12,250</td>
<td>1.210</td>
</tr>
<tr>
<td>LPS</td>
<td>24,222</td>
<td>2.400&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>Data expressed is the mean cpm of triplicate assays from the 3 animals in each group. SD did not exceed 10% of the mean. Only data of the dilution which gave the highest cpm is shown (1:8).

<sup>b</sup>The mean stimulation index (SI) was calculated by dividing the mean of each antigen cpm by the mean of the media control for each animal. SD did not exceed 10% of the mean.

<sup>*</sup>P<0.05, media comparison.

<sup>e</sup>P<0.05, group comparison.
TABLE 2. Mean Mouse Thymocyte Proliferation By Monocyte Supernatants In The Presence Of Con A After Sensitization

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Infected Group cpm&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Infected Group SI&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Noninfected Group cpm</th>
<th>Noninfected Group SI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Media</td>
<td>2,257</td>
<td>1.000</td>
<td>453&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1.000</td>
</tr>
<tr>
<td>johnin</td>
<td>1,683</td>
<td>0.776</td>
<td>6,136</td>
<td>13.552&lt;sup&gt;*e&lt;/sup&gt;</td>
</tr>
<tr>
<td>KLH</td>
<td>2,992</td>
<td>1.333</td>
<td>2,758</td>
<td>6.110&lt;sup&gt;*e&lt;/sup&gt;</td>
</tr>
<tr>
<td>PPD</td>
<td>3,110</td>
<td>1.412</td>
<td>3,075</td>
<td>6.790&lt;sup&gt;*e&lt;/sup&gt;</td>
</tr>
<tr>
<td>LPS</td>
<td>5,202</td>
<td>2.296</td>
<td>3,572</td>
<td>7.884&lt;sup&gt;*e&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>Data expressed is the mean cpm of triplicate assays form the 3 animals in each group. SD did not exceed 10% of the mean. Only data of the dilution which gave the highest cpm is shown (1:8).

<sup>b</sup>The mean stimulation index (SI) was calculated by dividing the mean of each antigen cpm by the mean of the media control for each animal. SD did not exceed 10% of the mean.

*<sup>p</sup><0.05, media comparison.

<sup>e</sup><sup>p</sup><0.05, group comparison.
LITERATURE CITED


Interleukin-2 (IL-2) production from peripheral blood mononuclear cells was measured in cattle naturally infected with *Mycobacterium paratuberculosis* and in healthy control cattle using a Concanavalin A induced blast cell proliferation assay. Production of IL-2 was significantly reduced in infected animals when peripheral blood mononuclear cells were stimulated with Con A. Animals were sensitized with *M. paratuberculosis* bacterin, live *M. bovis* BCG and keyhole limpet hemocyanin in Freund's incomplete adjuvant and IL-2 production re-evaluated. Infected animals showed no difference in IL-2 production after sensitization while healthy animals significantly increased production to the sensitizing antigens. These data indicate that cattle naturally infected with *Mycobacterium paratuberculosis* have a decreased ability to produce IL-2 in response to Con A and that sensitizing doses of mycobacterial and foreign antigens do not increase IL-2 production in response to
those antigens. Defective IL-2 production or the presence of suppressor substances may account for these observations and may contribute to disease persistence in these animals.

Introduction

Bovine paratuberculosis is a chronic enteric disease caused by infection with *Mycobacterium paratuberculosis* (*M. paraTB*). The disease is prevalent throughout the United States and results in significant economic losses. The host immune status with respect to production of soluble immuno-inflammatory agents (interleukins, interferons, prostanoids, etc.) has been ignored in paratuberculosis with the exception of in vitro assays such as migration inhibition and lymphocyte blastogenesis which measure cytokine production indirectly and not quantitatively. Quantitative analysis of these mediators should afford a better understanding of the disease and the immune defect responsible for persistence of infection.

Interleukin-2 (IL-2) is a soluble lymphokine which functions to enhance proliferative responses and clonal expansion of activated lymphocytes. Antigenic, mitogenic, or alloantigenic stimulation of lymphocytes results in IL-2 release and IL-2 receptor expression. Other serum factors such as
transferrin are required to drive IL-2 stimulated T-cells through the S-phase of growth.\textsuperscript{2,29} It is also IL-2 which promotes transferrin receptor expression on activated T-cells.\textsuperscript{2}

Maintenence of the proliferative response in activated T-cells serves as the basis for IL-2 assays.\textsuperscript{7} This feature has also been the important factor in recent advances in propagation of cloned T-cell lines with specific antigen reactivity and cells which selectively produce large amounts of specific lymphokines.\textsuperscript{3}

Bovine IL-2 has been characterized recently.\textsuperscript{21} Sodium dodecyl sulfate polyacrylamide gel electrophoresis and chromatofocusing revealed the presence of three active molecular species which support long-term growth of Con A or mixed lymphocyte reaction-activated bovine T-cells. Cortisol has also been shown to suppress IL-2 in the bovine both in vitro and in vivo.\textsuperscript{3} The following data represent an initial evaluation of IL-2 production in cattle with chronic paratuberculosis.

\textbf{Materials and Methods}

\textbf{Animals:}

Three adult Brahman-cross cattle diagnosed positive for bovine paratuberculosis by rectal biopsy, ileal biopsy and by enzyme-linked immunosorbent assay\textsuperscript{31} were donated to the Louisiana State University School of
Veterinary Medicine by local cattle producers. These animals had a history of weight loss and intermittent diarrhea of at least one year. In addition, 3 non-infected healthy cattle were selected at random from the School of Veterinary Medicine teaching herd. Fecal cultures from control animals using Herrold's egg-yolk media with and without 2 µg/ml mycobactin J (Allied Labs, Ames, IA) were negative after 6 months incubation. A single healthy adult cow was selected at random as a blast cell donor for all IL-2 assays.

Media and Reagents

The medium used for all cell cultures consisted of RPMI 1640 (GIBCO Laboratories, Chargin Falls, OH) supplemented with 4 mM L-glutamine and 10% fetal calf serum (FCS) without antibiotics (complete medium). Phosphate-buffered saline (PBS), pH = 7.4 was used as a washing solution. Concanavalin A (Con A), Keyhole limpet hemocyanin (KLH) (Sigma Chemical Co., St. Louis, MO), johnin and Mycobacterium bovis (M. bovis) PPD (Veterinary Services Laboratory, Ames, IA) were diluted to appropriate concentrations in complete media. Freshly prepared [³H]thymidine in complete media was used to assess proliferative responses. Animals from both groups were sensitized by subcutaneous injections of 800 mg KLH in Freund's incomplete adjuvant (Sigma Chemical Co., St.
Louis, MO), 0.5 ml heat-killed \textit{M. paraTB} bacterin (Fromm Laboratories, Grafton, WI) and $6 \times 10^8$ live \textit{M. bovis} BCG (Trudeau Institute, Saranac Lake, NY)

\textbf{Blood Mononuclear Cells}

Heparinized whole blood (40 ml) was collected by venapuncture and diluted 1:1 with PBS. An equal volume of Histopaque-1.083 (Sigma Chemical Co., St. Louis, MO) was slowly injected underneath the blood using a 6 in, 18 ga needle. The blood-Histopaque gradient was centrifuged at 1000 X g for 45 min and the mononuclear cell band at the interface was harvested and the cells washed three times with PBS. Platelet contamination was minimized by differential low speed centrifugation during washing (250 X g for 10 min). The cells were suspended in complete medium to a final concentration of $4 \times 10^6$/ml. Cell viability was >95\% using trypan blue exclusion.

\textbf{Preparation of IL-2 Containing Supernatants}

Preparation and assay of IL-2 containing supernatants were performed by the method of Oldham et al with some modification.\textsuperscript{22} Mononuclear cells (2 ml) were placed in wells of a 24 well flat bottom plate (Corning Glass Works, Corning, NY). The cells from each animal were cultured with 200 $\mu$l of one of the following; 500 $\mu$g/ml Con A, 100 $\mu$g/ml johnin, 100 $\mu$g/ml \textit{M. bovis} PPD, 100
μg/ml KLH and complete media. The cells were incubated in a humidified atmosphere for 1 hr at 37°C with 5% CO₂. The cell suspensions were harvested (200 X g for 10 min) and the cells washed once with PBS (200 X g for 10 min) and resuspended in complete medium at a concentration of 4 X 10⁶/ml. The wells were rinsed twice with 3 ml of PBS and the cells returned to their respective wells. The cells were incubated for 24 hrs under the same conditions as above and the supernatants harvested and maintained at -60°C until assayed (within 2 weeks).

**Preparation of Con A Blasts**

Peripheral blood mononuclear cells were collected as above and suspended to a final concentration of 2 X 10⁶/ml in complete media containing 5 μg/ml Con A. After incubation for 5 days under the same conditions as above, the cells were gently pelleted (200 X g for 10 min), washed once with PBS and suspended in complete media at a concentration of 2 X 10⁶/ml.

**IL-2 Assay**

The Con A blasts (100 μl) were placed in wells of a 96 well microtiter plate. Supernatants containing IL-2 were thawed and two-fold dilutions prepared to 1:64. The dilutions were added to triplicate wells containing the blast cells and incubated for 36 hrs. The cells were
pulsed with 0.5 μCi $[^3$H]thymidine and harvested onto glass fiber disks 18 hrs later using a semi-automated cell harvester (Skranton Inc., Sterling, VA). The disks were dried overnight at 60°C and placed in scintillation vials to which was added 3 ml of scintillation cocktail (Fluorolloy™, Beckman Instruments, Fullerton, CA). The vials were placed in the dark overnight to minimize inherent chemiluminescence. Counts were made for 2 min on a Packard Tri-Carb 4640 beta counter (Packard Instrument Co., Downers Grove, IL).

Statistical Analysis

Wilcoxon's rank sum test was used to identify differences using a dilution (1:4) which gave the highest blast cell proliferative response. A difference was considered significant at $P<0.05$.

Results

Con A blast proliferation induced by IL-2 containing supernatants was significantly ($P<0.05$) lower for infected cattle versus non-infected cattle with respect to Con A stimulation before sensitization (table 1), and after sensitization (table 2). The Con A, johnin, and PPD responses in the infected group was significantly ($P<0.05$) higher than the media control within that group before and after sensitization. Proliferative responses
obtained from non-infected and infected animals were highest with Con A stimulation. No significant (P>0.05) effect was seen with supernatants obtained by KLH stimulation in both groups before sensitization. The infected group showed significant differences between johnin and *M. bovis* PPD when compared to the non-infected group after sensitization. There was no significant (P>0.05) difference in the results obtained post-sensitization in the infected group (table 2). The non-infected cattle had significantly (P<0.05) increased IL-2 production in response to KLH, johnin, and *M. bovis* PPD after sensitization. No significant (P>0.05) alteration was observed in the Con A response after sensitization in the non-infected group. The KLH, johnin, PPD, and Con A responses were significantly (P<0.05) greater in the non-infected group in comparison to the infected group after sensitization. Sensitization was confirmed in the non-infected cattle by positive migration inhibition, lymphocyte stimulation, and skin test responses to the sensitizing antigens. The infected group failed to become sensitized based on the same assays (Chapter 2).

**Discussion**

In addition to T-cell proliferation, IL-2 has several other known functions such as induction of lymphokine activated killer cells which efficiently lyse a variety
of autologous and allogeneic tumor cells.\textsuperscript{24} Enhanced cytotoxicity of human monocytes has also been described as a function of IL-2.\textsuperscript{8,16} The ability of T-cells to produce IL-2 has not been previously investigated in bovine paratuberculosis. Comparisons of IL-2 production can be made with other mycobacterial diseases in which this lymphokine has been examined, such as in leprosy.\textsuperscript{10,13,19} Bovine paratuberculosis, like leprosy, often results in diffuse granulomatous disease.\textsuperscript{6,23} Lepromatous leprosy patients are known to have deficient T-cell responses to \textit{M. leprae} but not to other antigens while patients with tuberculoid leprosy have strong T-cell responses to \textit{M. leprae}.\textsuperscript{20,28} It was previously indicated that this unresponsiveness was related to a deficiency in IL-2 production and that proliferative responses to \textit{M. leprae} could be induced by an IL-2-rich T-cell conditioned media.\textsuperscript{10} This finding was disputed when it was demonstrated that failure of T-cells from lepromatous patients to respond to \textit{M. leprae} was due to a defective IL-2 receptor expression mechanism. Receptor expression was not reversed by IL-2 or IL-1 even though IL-1 had been shown to induce IL-2 receptor expression.\textsuperscript{13,19} Another study showed that T-cell unresponsiveness to \textit{M. leprae} could be reversed by incubation of the T-cells in simple media in the absence of \textit{M. leprae} antigen for 48
hrs. This phenomenon was explained by the induction of a CD4+ suppressor cell by *M. leprae*.

Cattle with paratuberculosis, in contrast to human patients with lepromatous leprosy, have positive, although variable, lymphocyte proliferative responses with in vitro exposure to johnin and other mycobacterial antigens.4,11,12 It is only in the terminal stages of the disease when non-specific anergy is present that indicators of cell mediated immunity such as skin reactivity are diminished.6 The criteria (weight loss and intermittent diarrhea of one year duration) used in this experiment to stage the animals with respect to disease spectrum does not reflect immune status of the host. Whether chronically infected and anergic animals or recently infected animals respond similarly with respect to IL-2 production is not known.

These data indicate that cattle with chronic bovine paratuberculosis have a decreased capacity to produce IL-2 in response to Con A and sensitizing antigens. Human IL-2 production can be inhibited by muramyl dipeptide, a known constituent of mycobacteria14,15 The mechanism of this suppression has been shown to be mediated by suppressor T-cells and prostaglandins, ie, PGE2.14,15 Muramyl dipeptide inhibition of Con A induced blood mononuclear cell proliferation was due at least in part to suppression of IL-2.14,15 The suppression of IL-2 in
cattle with paratuberculosis may be due to mycobacterial components such as muramyl dipeptide or other soluble products derived from the tremendous number of organisms present in vivo. Alternatively, these and other mycobacterial constituents or products may induce suppressor substances which inhibit lymphocyte reactivity as described for lepromatous leprosy^{25,26}.
Table 1. Mean Con A Blast Proliferative Responses By Blood Mononuclear Cell Supernatants Containing IL-2 Before Sensitization

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Infected cpmp</th>
<th>Infected SI</th>
<th>Non-Infected cpmp</th>
<th>Non-Infected SI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Media</td>
<td>750</td>
<td>1.00</td>
<td>976</td>
<td>1.00</td>
</tr>
<tr>
<td>PPD</td>
<td>3,773</td>
<td>5.03*</td>
<td>1,497</td>
<td>1.53</td>
</tr>
<tr>
<td>Johnin</td>
<td>4,477</td>
<td>5.96*</td>
<td>1,605</td>
<td>1.64</td>
</tr>
<tr>
<td>KLH</td>
<td>978</td>
<td>1.30</td>
<td>1,821</td>
<td>1.87</td>
</tr>
<tr>
<td>Con A</td>
<td>8,675</td>
<td>11.56*</td>
<td>30,331</td>
<td>31.08*</td>
</tr>
</tbody>
</table>

\( ^a \) Represents the mean cpm of triplicate assays from the 3 infected cattle. SD did not exceed 10% of the mean. Only data from the dilution which gave the highest cpm is shown (1:4).

\( ^b \) The mean stimulation index was calculated by dividing the mean of each antigen cpm by the mean of the media control from each animal.

\( ^*P<0.05, \) media comparison.

\( ^eP<0.05, \) group comparison.
Table 2. Mean Con A Blast Proliferative Responses By Blood Mononuclear Cell Supernatants Containing IL-2 After Sensitization

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Infected (cpm)</th>
<th>Infected (SI)</th>
<th>Non-Infected (cpm)</th>
<th>Non-Infected (SI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Media</td>
<td>1,116</td>
<td>1.00</td>
<td>835</td>
<td>1.00</td>
</tr>
<tr>
<td>PPD</td>
<td>5,267</td>
<td>4.72*</td>
<td>7,997</td>
<td>9.57*ε</td>
</tr>
<tr>
<td>johnin</td>
<td>6,003</td>
<td>5.38*</td>
<td>9,815</td>
<td>11.75*ε</td>
</tr>
<tr>
<td>KLH</td>
<td>1,139</td>
<td>1.02</td>
<td>10,363</td>
<td>12.41*ε</td>
</tr>
<tr>
<td>Con A</td>
<td>8,897</td>
<td>7.97*</td>
<td>22,570</td>
<td>27.03*ε</td>
</tr>
</tbody>
</table>

a Represents the mean cpm of triplicate assays from the 3 infected cattle. SD did not exceed 10% of the mean. Only data from the dilution which gave the highest cpm is shown (1:4).

b The mean stimulation index was calculated by dividing the mean of each antigen cpm by the mean the media control for each animal.

*P<0.05, media comparison.

εP<0.05, group comparison.
LITERATURE CITED


Abstract

Bovine transfer factor specific for Mycobacterium paratuberculosis and keyhole limpet hemocyanin was prepared from lymph node dialysates and administered to three cattle with chronic bovine paratuberculosis. Cutaneous delayed hypersensitivity, lymphocyte blastogenesis, migration inhibition, interleukin-1 release and interleukin-2 production were measured in response to M. bovis PPD, johnin, and keyhole limpet hemocyanin before and after treatment with the transfer factor. There was no change in cutaneous delayed hypersensitivity after transfer factor treatment. No alterations in histopathologic features of pre- and post-treatment sections of ileum were noted. Transfer factor treatment increased inhibition of migration of mononuclear cells and interleukin-2 production to johnin and KLH. There was no effect on lymphocyte blastogenesis and release of interleukin-1 by blood monocytes.
These data suggest that cattle with chronic bovine paratuberculosis may benefit from transfer factor therapy and that the effects of transfer factor are partially mediated by an increase in interleukin-2 production.

Introduction

Bovine paratuberculosis is a chronic wasting disease produced by enteric infection with *Mycobacterium paratuberculosis*.\(^{12}\) Persistence of disease results from inability of the infected host to rid itself of the infection. This implies that there is a defect in cell mediated immunity. Blood monocytes and monocyte-derived macrophages from non-infected cattle have been shown to be unable to effectively kill the organism and allow intracellular proliferation.\(^{3,48}\) A deficient T-cell response (decreased killer cell activity and interferon production) could explain why infected cattle cannot eliminate the organism. It has been suggested that cattle with paratuberculosis may benefit from transfer factor (TF) therapy.\(^{24}\)

Since the discovery of TF by Lawerence,\(^{26,27}\) viable leukocytes, leukocyte extracts, or dialysates of leukocyte extracts have been used to transfer delayed hypersensitivity to previously unsensitized individuals. Some of the clinical syndromes treated with transfer factor have included cancer immunotherapy,\(^{33,39}\) internal
parasitism, lupus erythematosus, sarcoidosis, vaccinia, coccidiomycosis, tuberculosis, lepromatous leprosy, and recently intestinal cryptosporidiosis in patients with human immunodeficiency virus infections. These treatment results have been variable, although often promising.

Leukocyte lysates which contain inducer factors and suppressor factors have been obtained from T-helper and T-suppressor cells, respectively. Much of the variability noted in clinical applications has probably been due to the fact that the crude lysates contained mixtures of both factors. The suppressor factor has been used for the modulation of diseases such as lupus erythematosis and autoimmune thrombocytopenic purpura characterized by an undesirable immune response.

Certain chemical and biological properties of TF have been well documented. In dialysates from human cells the active component is usually present in the 3.5 to 12kD molecular weight range, and has been shown to bind to specific antigen and anti-Ia antibody. The inducer factor is absorbed by T-suppressor cells and macrophages. Bovine TF specific for Mycobacterium tuberculosis PPD has been characterized as an oligoribonucleopeptide with a molecular weight of between 1.1 to 3.0kD. It has been postulated that TF may be a
dialyzable fragment of a T-helper cell receptor which equips the non-committed immunocyte with an antigen binding moiety.\textsuperscript{30}

The duration of effect of treatment with TF has not been well documented in every case. In a study using TF as a preventative measure for varicella-zoster virus infection, it was found that antibody titers of TF-treated patients developed after virus exposure and remained as high as those in infected individuals for 17 months.\textsuperscript{42} The TF-treated group also had an active cell mediated immunity for the same period of time as demonstrated in vitro and by skin tests.

The effect of TF has not been previously evaluated in bovine paratuberculosis, but its effect on other mycobacterial diseases has been examined. Lepromatous leprosy patients were evaluated using TF which was prepared from donors selected on the basis of positive skin reactivity and in vitro lymphocyte responses.\textsuperscript{7} Infected recipients were initially negative to skin tests with lepromin. After administration of TF the recipients became skin test positive after the first or third days.\textsuperscript{7} In vitro lymphocyte blastogenesis results were not altered. The recipients experienced erythema of the cutaneous lesions and occasionally fever. There was no alteration in histologic characteristics of the lesions when compared to pre-TF lesions. The majority of the
recipients reverted to a skin test negative status about 25 months after TF administration. These changes, although short-lived are extremely promising when it is considered that only one dose of TF was given. In patients with tuberculosis which was unresponsive to conventional tuberculostatic drugs, TF has been an important adjunct in therapy. 44,47

**Materials and Methods**

**Animals**

Three naturally infected Brahman cross-bred cattle were donated to the Louisiana State University Veterinary Teaching Hospital by Louisiana cattle producers. The cattle were confirmed as positive for paratuberculosis by ileal and mesenteric lymph node biopsies and positive ELISA test results.46 The cattle were housed on concrete and fed Bermuda grass hay ad libitum. Two healthy Brahman calves (180 kg body weight) were purchased from a local stockyard for production of transfer factor.
Experimental Design

Biopsies of the ileum and and mesenteric lymph nodes were taken from each animal and the surgical sites allowed to heal for at least three weeks. Assays were performed for IL-1, IL-2, migration inhibition factor (MIF), lymphocyte blastogenic (LB) responses and cutaneous hypersensitivity in response to KLH, M. bovis PPD and johnin prior to treatment with transfer factor. Transfer factor was administered weekly for three weeks. At the end of the treatment period all assays were repeated and necropsies performed.

Skin Testing

The cervical area of each cow was clipped and double skin thickness measured prior to injection. Intradermal injections of 0.2 ml containing 1000 μg johnin, M. bovis PPD and KLH in PBS were given to each cow. For a negative control PBS was used. The change in double skin thickness was recorded after 48 hrs. The cattle were retested using the contralateral cervical area after transfer factor treatment.

Media and Reagents

The medium used for most assays consisted of RPMI 1640 (GIBCO Laboratories, Chargin Falls, OH) supplemented with 4 mM L-glutamine and 10% fetal calf serum (FCS)
without antibiotics (complete culture medium, CCM). Dulbecco's MEM (GIBCO Laboratories, Chargin Falls, OH) was used in the migration inhibition assay. Phosphate-buffered saline (PBS), pH = 7.4, was used as a washing solution. Phytohemaglutinin (PHA), Cocanavalin A (ConA), keyhole limpet hemocyanin (KLH) (Sigma Chemical Co., St. Louis, MO), johnin and Mycobacterium bovis (M. bovis) PPD (Veterinary Services Laboratory, Ames, IA) were diluted to appropriate concentrations (as determined by previous dose response trials) in CCM or PBS. Freshly prepared $^3$H]thymidine in CCM was used to assess proliferative responses in IL-1, IL-2 and LB assays. Mycobacterium paratuberculosis bacterin (Fromm Laboratories, Grafton, WI) and KLH in Freund's incomplete adjuvant (Sigma Chemical Co., St. Louis, MO) were used to innoculate the donor calves for transfer factor preparation. All assays were performed before and after transfer factor treatment.

Blood Mononuclear Cells

Heparinized whole blood was collected by venapuncture and diluted 1:1 with (PBS). An equal volume of Histopaque-1.083 (Sigma Chemical Co., St. Louis, MO) was slowly injected underneath the blood using a 6 in, 18 ga needle. The blood-Histopaque gradient was centrifuged at 1000 X g for 45 min and the mononuclear cell band at
the interface was removed and the cells washed three times with PBS. Platelet contamination was minimized by differential low speed centrifugation during washing (250 X g for 10 min). The cells were suspended in complete medium to a final concentration of 2-5 X 10^6/ml depending on the assay to be performed. Cell viability was >95% using trypan blue exclusion.

Preparation of Adherent Cells for IL-1

Gradient-separated mononuclear cells were suspended in CCM at a concentration of 5 X 10^6/ml. The cells were incubated in a 75 cm^2 plastic culture flask (15 to 20 ml per flask) for 1 h. Non-adherent cells were removed by vigorously washing 5 times with PBS. Adherent cells were removed using a plastic cell scraper (Costar, Cambridge, MA) and suspended in CCM containing 2% FCS at a concentration of 1 X 10^6/ml (trypan blue exclusion showed a viability of >90%). The recovered cell suspension consisted of >95% monocytes with slight platelet contamination. Aliquots of adherent cells (1ml) were placed in wells of a 24 well plate (Corning Glass Works, Corning, NY).

Preparation of IL-1 Containing Supernatants:

To 1 ml aliquots of adherent cells from each animal
was added an equal volume of one the following; media with 2% FCS, 50 μg/ml johnin, 50 μg/ml M. bovis PPD, 50 μg/ml KLH, and 20 μg/ml LPS. Cells were cultured for 24 hrs at 37°C and 5% CO₂. Supernatants were harvested and maintained at -60°C until assayed (within 2 weeks).

IL-1 Assay:

The assay for IL-1 followed the guidelines as reviewed by Gearing, et al with minor modifications. Adherent cell supernatants were thawed and serial two-fold dilutions were made with media to 1:64. The thymuses from 6 wk old C3H/HeJ mice (Jackson Laboratories, Bar Harbor, ME) were used in all assays. Each thymus was aseptically removed and teased apart in 5 ml of CCM. The thymocytes and thymic tissue were allowed to sediment at 1 X g for ten minutes. About 4 ml of cell suspension were withdrawn and adjusted to a final concentration of 5 X 10⁶/ml in media containing 10% FCS and 1.5 μg/ml Con A. Viability assessed by trypan blue exclusion was >95%. Thymocytes in 100 μl of media were added to the wells of a 96 flatbottom well culture plate. An equal volume of diluted adherent cell supernatant was added to the thymocyte cultures. After incubation for 48 hrs, 0.5 μCi/50μl [³H]thymidine in CCM was added to each well. Cells were harvested after 18 hr onto glass fiber disks a semi-automated cell harvester (Skranton Inc.,
Sterling, VA). The disks were dried overnight at 60°C and placed in scintillation vials to which was added 3 ml of scintillation cocktail (Fluoralloy™, Beckman Instruments, Fullerton, CA). The vials were placed in the dark overnight to minimize inherent chemiluminescence. Counts were made for 2 min on a Packard Tri-Carb 4640 beta counter (Packard Instrument Co., Downers Grove, IL). All assays were performed in triplicate. Supernatants were intermittently screened for interleukin-2 as described below. Interleukin-2 activity using this assay was negligible.

Preparation of IL-2 Containing Supernatants

Preparation and assay of IL-2 containing supernatants were performed by the method of Oldham et al with minor modification. Mononuclear cells were suspended in CCM at a concentration of 4 X 10⁶/ml and 2 ml of cells were placed in wells of a 24 well plate (Corning Glass Works, Corning, NY). The cells from each animal were cultured with 200 µl CCM containing one of the following; 500 µg/ml Con A, 100 µg/ml johnin, 100 µg/ml M. bovis PPD, 100 µg/ml KLH or CCM without additive. The cells were incubated in a humidified atmosphere for 1 hr at 37°C with 5% CO₂. The cell suspensions were harvested and the cells washed once with PBS. The wells were rinsed twice with 3 ml of PBS and the cells returned to their
respective wells. The cells were incubated for 24 hrs under the same conditions as above and the supernatants were harvested and maintained at -60°C until assayed (within 2 weeks).

Preparation of Con A Blasts

Blood was collected from a single healthy cow and used to prepare Con A blasts for each IL-2 assay. Peripheral blood mononuclear cells were collected as above and suspended to a final concentration of 2 X 10^6/ml in CCM containing 5 µg/ml Con A. After incubation for 5 days under the same conditions as described above, the cells were gently pelleted (200 X g for 10 min), washed once with PBS and suspended in CCM at a concentration of 2 X 10^6/ml.

IL-2 Assay

The Con A blasts (100 µl) were placed in wells of a 96 well microtiter plate. Supernatants containing IL-2 were thawed and two-fold dilutions prepared to 1:64. The dilutions were added to triplicate wells containing the blast cells and incubated for 36 hrs. The cells were pulsed with 0.5 µCi [³H]thymidine and harvested after 18 hrs incubation. Scintillation counting was done as described above.
Lymphocyte Blastogenesis

Gradient-separated mononuclear cells were suspended in CCM at a concentration of 2 X 10^6/ml and 100 µl aliquots were placed in the wells of a 96 flatbottom well plate. To triplicate wells was added 100 µl volumes of CCM with one of the following: 50 µg/ml johnin, 50 µg/ml M. bovis PPD, 30 µg/ml KLH, 25 µg/ml PHA, or CCM without additives. The cells were incubated for 72 hrs., pulsed with 0.5 µCi/50µl [3]thymidine, and collected after 18 hrs and handled as described above.

Migration Inhibition Assay

A microagarose droplet assay as described by Harrington and Stastny was used with minor modifications.19 Cell pellets containing 10^8 gradient-separated mononuclear cells were combined with 100 µl of 2 X Dulbecco's MEM with 20% FCS and 100 µl of 0.4% agarose in distilled water. The cells, media and agarose were gently mixed and 1 µl droplets were placed in the center of the wells of a 96 flatbottom well plate. The plate was refrigerated (4°C) for 10 min prior to the addition of antigens. To triplicate wells 100 µl volumes CCM was added containing one of the following: 50 µg/ml johnin, 50 µg/ml M. bovis PPD, 50 µg/ml KLH, 25 µg/ml PHA and CCM without additive. After 48 hrs incubation each well was photographed and a 5 X 7 print made. The two
most symmetrical droplets from each treatment were selected and the distance was measured from the edge of the droplet to the edge of the cell migration zone. Three random measurements were made from each of the selected droplets and the mean and standard deviation were calculated. Data were expressed as % inhibition of migration: 

\[
\frac{\text{mean migration distance of media control} - \left( \frac{\text{mean migration distance of antigen}}{\text{mean migration distance of media control}} \right)}{100}
\]

A 20% inhibition of migration was considered a positive MIF assay reaction.11

**Preparation and Administration of Crude TF**

Two calves were inoculated with 8.0 mg KLH in Freund's incomplete adjuvant and 1 ml *Mycobacterium paratuberculosis* bacterin (twice the manufacturers recommended dose). After three weeks, sensitization was confirmed by positive (>5 mm increase) skin test reactivity to johnin and KLH. The calves were killed and prescapular, submandibular, prefemoral, popliteal, cervical, tracheobronchial, mesenteric, perirenal, and pelvic lymph nodes removed. The lymph nodes were minced and pressed through a 40 mesh screen. The resulting cell suspension (5 X 10^8/ml) was incubated in Hank's balanced salt solution for 4 hrs at which time the suspension became acidic. The supernatants were harvested and an Amicon DC-10 hollow fiber concentrator in dialysis mode
was used to collect the fraction which contained material of 10kD molecular weight and less (designated crude TF). This fraction was frozen in 10 ml aliquots and maintained at -60°C until use (within 5 weeks). Each infected animal was given 10 ml of crude TF subcutaneously at weekly intervals for three weeks.

Necropsy

Treated animals were killed and sections of intestine adjacent to the original biopsy site and a regional mesenteric lymph node were fixed in 10% neutral buffered formalin, embedded in methacrylate, sectioned, mounted on glass slides, and examined by light microscopy. Hematoxylin and eosin and acid-fast stained sections were examined in pre- and post-treatment tissues.

Statistics

Wilcoxon's rank sum test was used to compare data for the IL-1 and IL-2 assays. Lymphocyte blastogenesis, migration inhibition, and skin test data were compared with Student's t-test. Results were considered significant at P<0.05.

Results

The administration of TF had no effect on skin test
reactivity (P>0.05) in this experiment (table 1). Migration inhibition and IL-2 production were significantly increased (P<0.05) using KLH (figure 1) and johnin (tables 2 and 3, respectively). There was no difference in LB responses with johnin, KLH, and PHA before or after TF treatment (table 4). The high blastogenic responses and inhibition of migration in response to *M. bovis* PPD noted before TF treatment were significantly (P<0.05) reduced (tables 2 and 4). Increases (P<0.05) in IL-2 production were seen only with johnin and KLH, with no change in response to Con A and *M. bovis* were not affected. Production of IL-1 was unaffected (P>0.05) by administration of TF (table 5). The cpm values and stimulation indices for IL-1 release were similar to those obtained previously (Chapter 3). Sections of ileum and mesenteric lymph node were similar before and after TF treatment. Both contained a marked granulomatous reaction in the lamina propria and submucosa. Moderate to marked numbers of acid-fast bacilli were present in macrophages and multinucleated giant cells (figures 2-5).

**Discussion**

The classic response of TF treatment is the transfer of cutaneous hypersensitivity to previously non-responsive individuals. Mazahari et al have shown
that positive cutaneous delayed hypersensitivity in primates may no be apparent until at least 21 days after administration of TF although in vitro migration inhibition was positive between 2 and 14 days.\textsuperscript{36} This may account for negative skin test results in this experiment as skin test reactions were examined only at 21 days after the onset of TF administration.

In general, lymphocyte blastogenesis and monocyte migration inhibition have been considered good correlates of in vivo cell mediated immunity transferred by TF\textsuperscript{16,35,40}. Some studies, however, indicate this not to be true.\textsuperscript{17,41} There has been poor agreement between lymphocyte blastogenesis and cutaneous hypersensitivity tests in TF recipients.\textsuperscript{10,16} Usually there is a positive skin test response with no alteration in lymphocyte transformation. Non-specific activities of TF which affect lymphocyte blastogenesis include augmentation or suppression of responses to antigens or mitogens.\textsuperscript{1,8,9,18,32,43}

It is interesting to note that in this experiment a previously significant blastogenic response to \textit{M. bovis} PPD was selectively suppressed after TF treatment even though the TF was not prepared from a known \textit{M. bovis} responsive donor. Monocyte migration inhibition assays are usually considered a more appropriate in vitro assay for the evaluation of the TF effects,\textsuperscript{22} however some
reports indicate the unfractionated (crude) TF-containing preparations augment non-specific production of migration inhibition factor.\textsuperscript{2,25}

Indirect evidence indicates that the T-cell is the target cell for TF.\textsuperscript{23,30} As the effector cell in cell mediated immune responses is the monocyte or macrophage, it would be reasonable to expect that TF acting on the T-cell would modulate the monocyte and macrophage. Transfer factor preparations have also been shown to elevate interferon levels.\textsuperscript{13} This may be the important communication link between the TF activated lymphocyte and the macrophage.

The ability of TF preparations to augment or suppress production of IL-1 and IL-2 has not been previously examined. The results of this experiment indicate that TF augments IL-2 production in response to specific antigens but does not increase the release of IL-1. A generalized (non-specific) increase in IL-2 production may account for the increased dermal hypersensitivity seen following TF administration, but would not account for antigen specificity. The increase in IL-2 noted in this experiment was seen only in response to antigens to which the TF was initially prepared.

The results of this study demonstrate a previously unreported effect of TF, an increased antigen-specific production of IL-2. Although cutaneous hypersensitivity
was not transferred and no lesion alterations were noted after treatment, the positive responses (increased IL-2 and greater migration inhibition) indicate that cattle with chronic bovine paratuberculosis may benefit from a longer duration of treatment with specific TF.
Table 1. Delayed Cutaneous Hypersensitivity in *Mycobacterium paratuberculosis*-Infected Cattle Treated With Transfer Factor

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Pre-Treatment</th>
<th>Post-Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Change in Skin Thickness (mm)</td>
<td>Change in Skin Thickness (mm)</td>
</tr>
<tr>
<td></td>
<td>Cattle #1</td>
<td>#2</td>
</tr>
<tr>
<td>PPD</td>
<td>4.2</td>
<td>3.2</td>
</tr>
<tr>
<td>johnin</td>
<td>5.3</td>
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</tr>
<tr>
<td>KLH</td>
<td>0.2</td>
<td>0.6</td>
</tr>
<tr>
<td>media</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Mean+SD

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Pre-Treatment</th>
<th>Post-Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPD</td>
<td>3.93±0.64</td>
<td>3.83±0.76</td>
</tr>
<tr>
<td>johnin</td>
<td>5.03±1.72</td>
<td>4.70±1.31</td>
</tr>
<tr>
<td>KLH</td>
<td>0.73±0.61</td>
<td>0.67±0.40</td>
</tr>
<tr>
<td>media</td>
<td>0.10±0.17</td>
<td>0.07±0.06</td>
</tr>
</tbody>
</table>

No significant difference after transfer factor treatment.
Table 2. Mean Percent Inhibition of Migration
in *Mycobacterium paratuberculosis*-Infected Cattle
Treated with Transfer Factor

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Pre-Treatment</th>
<th>Post-Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPD&lt;sup&gt;b&lt;/sup&gt;</td>
<td>43.2 ± 5.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>21.3 ± 1.8</td>
</tr>
<tr>
<td>johnin</td>
<td>33.7 ± 1.8</td>
<td>58.1 ± 7.1*</td>
</tr>
<tr>
<td>KLH&lt;sup&gt;c&lt;/sup&gt;</td>
<td>11.5 ± 0.9</td>
<td>52.5 ± 6.2*</td>
</tr>
</tbody>
</table>

<sup>a</sup>Mean±SD.

<sup>b</sup>Derived from *M. bovis*.

<sup>c</sup>Keyhole limpet hemocyanin.

*P<0.05
Table 3. Mean Con A Blast Proliferative Responses By Blood Mononuclear Cell Supernatants Containing IL-2 From *Mycobacterium paratuberculosis*-Infected Cattle Treated With Transfer Factor

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Pre-Treatment</th>
<th>Post-Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cpm&lt;sup&gt;a&lt;/sup&gt;</td>
<td>SI&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>Con A</td>
<td>7,612</td>
<td>15.02&lt;sup&gt;*&lt;/sup&gt;</td>
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</tbody>
</table>

<sup>a</sup>Represents the mean cpm of triplicate assays from the 3 infected cattle. SD is not greater than 10% of the mean. Only data from the dilution which gave the highest cpm is shown (1:4).

<sup>b</sup>The mean stimulation index was calculated by dividing the mean of each antigen cpm by the mean of the media control for each animal.

<sup>*</sup><i>P</i>&lt;0.05, media comparison.

<sup>ε</sup><i>P</i>&lt;0.05, group comparison.
Table 4. Mean Lymphocyte Blastogenesis in *Mycobacterium paratuberculosis* Infected Cattle Treated With Transfer Factor

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Pre-treatment</th>
<th>Post-treatment</th>
</tr>
</thead>
<tbody>
<tr>
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<td>cpm</td>
<td>SI$^a$</td>
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<td>media</td>
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<tr>
<td>PPD$^b$</td>
<td>10,627</td>
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<td>johnin</td>
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<td>KLH$^c$</td>
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</tr>
<tr>
<td>PHA$^d$</td>
<td>20,396</td>
<td>23.3</td>
</tr>
</tbody>
</table>

$^a$The mean SI was calculated by dividing the mean antigen cpm of triplicate assays from each of the 3 infected animals by the cpm of the media control. SD did not exceed 10% of the mean.

$^b$Derived from *M. bovis*; $^c$keyhole limpet hemocyanin; $^d$phytohemaglutinin.

All antigen and mitogen responses are significantly different than the media control ($P<0.05$). No differences are apparent between pre- and post-treatment data.
Table 5. Mean Mouse Thymocyte Proliferation By Monocyte Supernatants Containing IL-1 In The Presence Of Con A From *Mycobacterium paratuberculosis*-Infected Cattle Treated With Transfer Factor

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Pre-treatment</th>
<th>Post-treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cpm&lt;sup&gt;a&lt;/sup&gt;</td>
<td>SI&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>Media</td>
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<td>PPD</td>
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<td>johnin</td>
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<td>1.20</td>
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<td>KLH</td>
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<tr>
<td>LPS</td>
<td>22,701</td>
<td>2.39&lt;sup&gt;*&lt;/sup&gt;</td>
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</tbody>
</table>

<sup>a</sup>Data expressed is the mean cpm of triplicate assays from the 3 animals in each group. SD is not greater than 10% of the mean. Only data of the dilution which gave the highest cpm is shown (1:8).

<sup>b</sup>The mean stimulation index was calculated by dividing the mean of each antigen cpm by the mean the media control for each animal. SD did not exceed 10% of the mean.

<sup>*</sup>P<0.05, media comparison.
Figure 1. Example of increased migration inhibition in response to keyhole limpet hemocyanin after transfer factor treatment. Media control (top); before treatment (center); after treatment (bottom).
Figure 2. Ileal biopsy specimen taken before transfer factor treatment. There is a marked accumulation of macrophages in the lamina propria and submucosa. HE, 200X.
Figure 3. Section of ileum taken adjacent to the initial biopsy site after transfer factor treatment. Lesions noted are similar to the pre-treatment section. HE, 200X.
Figure 4. Lymph node biopsy specimen taken before transfer factor treatment. There is a moderate to marked accumulation of macrophages in the cortex. HE, 200X.
Figure 5. Mesenteric lymph node specimen taken adjacent to the initial biopsy site after transfer factor treatment. The section is similar to the pre-treatment biopsy sample.
LITERATURE CITED


CHAPTER 6

THE EFFECTS OF RIFABUTIN (ANSAMYCIN LM 427) IN CATTLE WITH CHRONIC BOVINE PARATUBERCULOSIS

Abstract

Three cattle with chronic paratuberculosis were examined for delayed cutaneous hypersensitivity, macrophage migration inhibition, lymphocyte blastogenesis, interleukin-1 production and interleukin-2 production in response to johnin, M. bovis PPD, and Keyhole limpet hemocyanin before and after treatment with rifabutin. Rifabutin was administered orally at a dose of 1200 mg daily for 3 weeks. Sections of ileum and mesenteric lymph node were examined microscopically before and after treatment. By the end of the treatment period there was a significant increase in skin reactivity to johnin. No changes in skin reactivity were seen with the other antigens. There was no difference in interleukin-1 release, interleukin-2 production, blastogenesis and migration inhibition before and after treatment.
Introduction

Bovine paratuberculosis is a chronic enteric infection caused by *Mycobacterium paratuberculosis* which results in diarrhea, weight loss and eventually death.² Treatment of affected cattle with antimycobacterial agents, such as clofazimine, has resulted in drug dependent remission of clinical signs, however, the cattle continued to shed the organism in feces even with prolonged duration of treatment (over 300 days)⁷,⁸,¹¹,¹⁹. Although a cure does not result from the administration of clofazimine, it may be useful for the treatment of valuable breeding stock.

Rifabutin (4-deoxo-3,4-[2-spiro-(N-isobutyl-4-piperidyl)](1H)-imidazole(2,5-dihydro)-rifamycin S), a semi-synthetic derivative of rifamycin S, was first synthesized by Marsili et al.⁹ This agent and other related compounds were found to have activity against *Mycobacterium tuberculosis* and *Mycobacterium avium-*complex organisms. It also has the advantage of a longer half-life than other derivatives of rifamycin S, such as rifampin.¹² Rifabutin has a broad spectrum of activity against gram-positive and gram-negative bacteria similar to rifampin.¹⁵ The compound has activity against rifampin-resistant strains of *Mycobacterium tuberculosis* presumably due to the difference in mechanism of action. Rifabutin interferes with DNA synthesis whereas rifampin
inhibits DNA-dependent RNA polymerase.\textsuperscript{18}

The in vivo use of Rifabutin has been primarily limited to experimental infections and AIDS patients concurrently infected with mycobacteria.\textsuperscript{5,6,12,14} Recently, a colony of stumptail macaques was diagnosed as having \textit{Mycobacterium paratuberculosis} infection with lesions similar to ruminant paratuberculosis.\textsuperscript{10} Macaques treated with rifabutin showed dramatic improvement and treated animals remained in clinical remission for up to 30 months post-treatment.

Rifabutin has not been previously evaluated as a potential therapeutic agent for bovine paratuberculosis. The purpose of this study was to evaluate the use of rifabutin in bovine paratuberculosis by the comparison of immunologic parameters and intestinal lesions after treatment.

\textbf{Materials and Methods}

\textbf{Animals}

Three naturally infected Brahman cross-bred cattle were donated to the Louisiana State University Veterinary Teaching Hospital by local cattle producers. The cattle were confirmed positive for paratuberculosis by ileal and mesenteric lymph node biopsies and positive ELISA test results.\textsuperscript{20} The cattle were housed on concrete and fed Bermuda grass hay ad libitum.
Experimental Design

Biopsies of the ileum and mesenteric lymph nodes were taken from each animal and surgical sites allowed to heal for at least three weeks. Assays were performed for interleukin-1 (IL-1), interleukin-2 (IL-2), migration inhibition factor (MIF), lymphocyte blastogenic (LB) responses and cutaneous hypersensitivity in response to KLH, M. bovis PPD and johnin prior to treatment with rifabutin. Rifabutin was administered daily per os for three weeks. At the end of the treatment period the assays were repeated and necropsies performed.

Media and Reagents

The culture medium used for most assays consisted of RPMI 1640 (GIBCO Laboratories, Chargin Falls, OH) supplemented with 4 mM L-glutamine and 10% fetal calf serum (FCS) without antibiotics (complete culture medium, CCM). Dulbecco's MEM (GIBCO Laboratories, Chargin Falls, OH) was used in the MIF assay. Phosphate-buffered saline (PBS), pH = 7.4, was used as a washing solution. Phytohemaglutinin (PHA), Concanavalin A (Con A), keyhole limpet hemocyanin (KLH) (Sigma Chemical Co., St. Louis, MO), johnin and Mycobacterium bovis (M. bovis) PPD (Veterinary Services Laboratory, Ames, IA) were diluted
to appropriate concentrations (as determined by previous dose response trials) in CCM or PBS. Freshly prepared $[^3H]$thymidine in CCM was used to assess proliferative responses in IL-1, IL-2 and LB assays. Rifabutin was a gift obtained from Adria Laboratories, Columbus, Ohio (Vernon Verhoeff).

Skin Testing

The cervical area of each cow was clipped and double skin thickness measured prior to injection. Intradermal injections of 0.2 ml containing 1000 μg johnin, M. bovis PPD and KLH in PBS were given to each cow. For a negative control PBS was used. The change in double skin thickness was recorded after 48 hrs. The cattle were retested using the contralateral cervical area after transfer factor treatment.

Blood Mononuclear Cells

Heparinized whole blood was collected by venapuncture and diluted 1:1 with (PBS). An equal volume of Histopaque-1.083 (Sigma Chemical Co., St. Louis, MO) was slowly injected underneath the blood using a 6 in, 18 ga needle. The blood-Histopaque gradient was centrifuged at 1000 X g for 45 min and the mononuclear cell band at the interface was removed and the cells washed three times with PBS. Platelet contamination was minimized by
differential low-speed centrifugation during washing (250 X g for 10 min). The cells were suspended CCM to a final concentration of 2-5 X 10^6/ml depending on the assay to be performed. Cell viability was >95% using trypan blue exclusion.

**Preparation of Adherent Cells for IL-1**

Gradient-separated mononuclear cells were suspended in CCM at a concentration of 5 X 10^6/ml. The cells were incubated in a 75 cm^2 plastic culture flask (15 to 20 ml per flask) for 1 h. Non-adherent cells were removed by vigorously washing 5 times with PBS. Adherent cells were removed using a plastic cell scraper (Costar, Cambridge, MA) and suspended in CCM containing 2% FCS at a concentration of 1 X 10^6/ml (trypan blue exclusion showed a viability of >90%). The recovered cell suspension consisted of >95% monocytes with slight platelet contamination. Aliquots of adherent cells (1ml) were placed in wells of a 24 well plate (Corning Glass Works, Corning, NY).

**Preparation of IL-1 Containing Supernatants:**

To 1 ml aliquots of 1 X 10^6 adherent cells/ml from each animal was added an equal volume of one of the following; CCM with 2% FCS, 50 μg/ml johnin, 50 μg/ml *M. bovis* PPD, 50 μg/ml KLH, and 20 μg/ml LPS. Cells were
cultured for 24 hrs at 37°C with 5% CO₂ and 100% humidity. Supernatants were harvested and maintained at -60°C until assayed (within 2 weeks).

**IL-1 Assay:**

The assay for IL-1 followed the guidelines as reviewed by Gearing, et al with minor modifications. Adherent cell supernatants were thawed and serial two-fold dilutions were made with CCM to 1:64. The thymuses from 6 wk old C3H/HeJ mice (Jackson Laboratories, Bar Harbor, ME) were used in all assays. Each thymus was aseptically removed and teased apart in 5 ml of CCM. The thymocytes and thymic tissue were allowed to sediment at 1 X g for ten minutes. About 4 ml of cell suspension was withdrawn and adjusted to a final concentration of 5 X 10⁶/ml in CCM containing 1.5 μg/ml Con A. Viability assessed by trypan blue exclusion was >95%. Thymocytes in 100 μl of CCM were added to the wells of a 96 flatbottom culture plate. An equal volume of diluted adherent cell supernatant was added to the thymocyte cultures. After incubation for 48 hrs, 0.5 μCi/50μl [³H]thymidine in CCM was added to each well. Cells were harvested after 18 hr onto glass fiber disks using a semi-automated cell harvester (Skranton Inc., Sterling, VA). The disks were dried overnight at 60°C and placed in scintillation vials to which was added 3 ml of
scintillation cocktail (Fluoralloy™, Beckman Instruments, Fullerton, CA). The vials were placed in the dark overnight to minimize inherent chemiluminescence. Counts were made for 2 min on a Packard Tri-Carb 4640 beta counter (Packard Instrument Co., Downers Grove, IL). All assays were performed in triplicate. Supernatants were intermittently screened for IL-2 as described below.

Preparation of IL-2 Containing Supernatants

Preparation and assay of IL-2 containing supernatants were performed by the method of Oldham et al. with minor modification. Mononuclear cells were suspended in CCM at a concentration of 4 X 10^6/ml and 2 ml of cells were placed in wells of a 24 well plate (Corning Glass Works, Corning, NY). The cells from each animal were cultured with 200 µl CCM containing one of the following; 500 µg/ml Con A, 100 µg/ml johnin, 100 µg/ml M. bovis PPD, 100 µg/ml KLH or CCM without additive. The cells were incubated in a humidified atmosphere for 1 hr at 37°C with 5% CO₂. The cell suspensions were harvested and the cells washed once with PBS. The wells were rinsed twice with 3 ml of PBS and the cells returned to their respective wells. The cells were incubated for 24 hrs under the same conditions as above and the supernatants
harvested and maintained at -60°C until assayed (within 2 weeks).

**Preparation of Con A Blasts**

Blood was collected from a single healthy cow and used to prepare Con A blasts for each IL-2 assay. Peripheral blood mononuclear cells were collected as above and suspended to a final concentration of 2 X 10^6/ml in CCM containing 5 μg/ml Con A. After incubation for 5 days under the same conditions as above, the cells were gently pelleted (200 X g for 10 min), washed once with PBS and suspended in CCM at a concentration of 2 X 10^6/ml.

**IL-2 Assay**

The Con A blasts (100 μl) were placed in wells of a 96 well microtiter plate. Supernatants containing IL-2 were thawed and two-fold dilutions prepared to 1:64. The dilutions were added to triplicate wells containing the blast cells and incubated for 36 hrs. The cells were pulsed with 0.5 μCi [³H]thymidine and harvested after 18 hrs and prepared for beta counting as before.

**Lymphocyte Blastogenesis**

Gradient-separated mononuclear cells were suspended in CCM at a concentration of 2 X 10^6/ml and 100 μl
aliquots were placed in the wells of a 96 well flat bottom microtiter plate. To triplicate wells was added 100 μl CCM with one of the following: 50 μg/ml johnin, 50 μg/ml M. bovis PPD, 30 μg/ml KLH, 25 μg/ml PHA, and CCM without additive. The cells were incubated for 72 hrs., pulsed with 0.5 μCi/50μl [3H]thymidine, and collected after 18 hrs and handled as described (Chapter 2).

**Migration Inhibition Assay**

A microagarose droplet assay as described by Harrington and Stastny was used with minor modifications.4 Cell pellets containing 10^8 gradient-separated mononuclear cells was add 100 μl of 2 X Dulbecco's MEM with 20% FCS and 100 μl melted agarose (SeaPlaque™, FMC Corporation, Rockland, ME) The cells, media and agarose were gently mixed and 1 μl droplets were placed in the center of the wells of a 96 flatbottom microtiter plate. The plate was refrigerated (4°C) for 10 min prior to the addition of antigens. To triplicate wells 100 μl CCM was added containing one of the following: 50 μg/ml johnin, 50 μg/ml M. bovis PPD, 50 μg/ml KLH, 25 μg/ml PHA and CCM without additive. After 48 hrs incubation each well was photographed and a 5 X 7 print was made. The two most symmetrical droplets from each treatment were selected and the distance from the edge of the droplet to the edge of the cell migration
zone was measured. Three random measurements were made from each of the selected droplets and the mean and standard deviation were calculated. Data were expressed as % inhibition of migration: 

\[
\left( \frac{\text{mean migration distance of media control} - \text{mean migration distance of antigen}}{\text{mean migration distance of media control}} \right) \times 100.
\]

A 20% inhibition of migration was considered a positive test.

**Administration of Rifabutin**

The cattle were each given 1200 mg rifabutin orally in gelatin capsules once daily for 21 days.

**Necropsy**

Treated animals were killed and sections of intestine adjacent to the original biopsy site and a regional mesenteric lymph node were fixed in 10% neutral buffered formalin, embedded in methacrylate, sectioned, mounted on glass slides, and examined by light microscopy. Hematoxylin and eosin and acid-fast stained sections were examined in pre- and post-treatment tissues.

**Statistics**

IL-1 and IL-2 production was compared using Wilcoxon's rank sum test. Migration inhibition, lymphocyte blastogenesis, and skin test data were
compared with Student's t-test. Results were considered significant at P<0.05.

Results

The delayed cutaneous hypersensitivity reaction to johnin significantly (P<0.05) increased after rifabutin treatment with no difference (P>0.05) in response to the other antigens (table 1). There was a slight increase (P>0.05) in IL-2 production to johnin stimulation, but no change was noted with the other antigens (table 2). No difference (P>0.05) in IL-1 release was observed before and after treatment (table 3). The cpm values and stimulation indices were similar to those previously obtained (chapter 3). There was a slight increase (P>0.05) in lymphocyte blastogenic responses with johnin (table 4). No alterations were found in migration inhibition before and after treatment (table 5). No histologic alterations were seen in sections of ileum and mesenteric lymph node after treatment (Figures 1-4). A marked granulomatous inflammatory reaction in the lamina propria and submucosa was observed. Macrophages and multinucleated giant cells contained variable numbers of acid-fast bacilli.

Discussion

Treatment with rifabutin produced a positive increase
in cutaneous delayed hypersensitivity to johnin. A possible explanation for this increase is that perhaps there was an increase in mycobacterial death releasing internal bacterial antigens important in delayed hypersensitivity reactions. An increase in bacterial death could not be substantiated on the basis of the tissue sections examined, because only pre- and post-treatment ileum and mesenteric lymph node sections were examined. The extent of tissue involvement before treatment was not determined. A second explanation may be that rifabutin itself increased the cutaneous response by directly affecting lymphocyte or accessory cell function. Lastly, it may be that rifabutin had a bacteriostatic effect resulting in a decrease in production of soluble substances from mycobacteria which inhibit skin test reactivity.

The trend of increased lymphocyte blastogenic responses to johnin may be a reflection of a similar trend in IL-2 production since IL-2 is known to increase T-cell proliferation in response to mitogen. This trend appears to suggest that cell-mediated immune responses are being partially restored. Additional trials with rifabutin in cattle with paratuberculosis may help to validate this observation.

The duration and dosage of rifabutin in this experiment was not sufficient to alter intestinal
and mesenteric lymph node lesions. A qualitative assessment indicated a granulomatous inflammatory response with the presence of numerous acid-fast bacilli similar to pre-treatment biopsy specimens.

The absorption, deposition and pharmacokinetics of rifabutin in the ruminant are not known. These parameters should also be investigated in order to more clearly define the value of rifabutin in the treatment of bovine paratuberculosis.
Table 1. Delayed Cutaneous Hypersensitivity
In *Mycobacterium paratuberculosis*-Infected Cattle
Treated With Rifabutin

<table>
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<tr>
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<th>Pre-Treatment</th>
<th>Post-Treatment</th>
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<td>#1  #2  #3</td>
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<td>PPD</td>
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<td>johnin</td>
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<td>10.4 8.8 7.5</td>
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<td>KLH</td>
<td>0.0 0.9 0.0</td>
<td>0.3 0.2 0.4</td>
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<td>media</td>
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<td>0.0 0.2 0.0</td>
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<table>
<thead>
<tr>
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<th>mean±SD</th>
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<tr>
<td>PPD</td>
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<td>3.93±0.95</td>
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<td>johnin</td>
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<td>8.90±1.45*</td>
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<td>KLH</td>
<td>0.30±0.52</td>
<td>0.30±0.10</td>
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<tr>
<td>media</td>
<td>0.03±0.06</td>
<td>0.06±0.12</td>
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*P<0.05
Table 2. Mean Con A Blast Proliferative Responses By Blood Mononuclear Cell Supernatants Containing IL-2 From Mycobacterium paratuberculosis-Infected Cattle Treated With Rifabutin

<table>
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<th>Post-Treatment</th>
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</thead>
<tbody>
<tr>
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<td>cpm&lt;sup&gt;a&lt;/sup&gt;</td>
<td>SI&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>Media</td>
<td>980</td>
<td>1.00</td>
</tr>
<tr>
<td>PPD</td>
<td>2,341</td>
<td>2.44&lt;sup&gt;*&lt;/sup&gt;</td>
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<tr>
<td>johnin</td>
<td>3,870</td>
<td>3.89&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>KLH</td>
<td>1,015</td>
<td>1.13</td>
</tr>
<tr>
<td>Con A</td>
<td>9,578</td>
<td>8.79&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>Represents the mean cpm of triplicate assays from the 3 infected cattle. SD is not greater than 10% of the mean. Only data from the dilution which gave the highest cpm is shown (1:4).

<sup>b</sup>The mean stimulation index was calculated by dividing the mean of each antigen cpm by the mean of the media control from each animal.

<sup>*</sup>P<0.05, media comparison.
Table 3. Mean Mouse Thymocyte Proliferation By Monocyte Supernatants Containing IL-1 In The Presence of Con A From *Mycobacterium paratuberculosis*-Infected Cattle Treated with Rifabutin

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Pre-treatment</th>
<th>Post-treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cpm&lt;sup&gt;a&lt;/sup&gt;</td>
<td>SI&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Media</td>
<td>12,654</td>
<td>1.00</td>
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<tr>
<td>PPD</td>
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<td>Johnin</td>
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<td>KLH</td>
<td>10,512</td>
<td>0.93</td>
</tr>
<tr>
<td>LPS</td>
<td>25,539</td>
<td>2.13&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>Data expressed is the mean cpm of triplicate assays form the 3 animals in each group. SD is not greater than 10% of the mean. Only data of the dilution which gave the highest cpm is shown (1:8).

<sup>b</sup>The mean lation index (SI) was calculated by dividing the mean of each antigen cpm by the mean of the media control from each animal.

<sup>*</sup>P<0.05, media comparison.
Table 4. Mean Lymphocyte Blastogenesis in *Mycobacterium partuberculosis* Infected Cattle Treated With Rifabutin

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Pre-treatment</th>
<th>Post-treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cpm</td>
<td>SI&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>media</td>
<td>1,008</td>
<td>1.0</td>
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<tr>
<td>PPD&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12,499</td>
<td>12.8</td>
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<tr>
<td>johnin</td>
<td>10,886</td>
<td>10.3</td>
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<td>KLH&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3,326</td>
<td>3.6</td>
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<td>PHA&lt;sup&gt;d&lt;/sup&gt;</td>
<td>18,547</td>
<td>19.1</td>
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</tbody>
</table>

<sup>a</sup>The mean SI was calculated by dividing the mean antigen cpm of triplicate assays from each of the 3 infected animals by the cpm of the media control. SD did not exceed 10% of the mean.

<sup>b</sup>Derived from *M. bovis*; <sup>c</sup>keyhole limpet hemocyanin; <sup>d</sup>phytohemaglutinin.

All antigen and mitogen responses are significantly different than the media control (P<0.05). No differences are apparent between pre- and post-treatment data.
Table 5. Mean Percent Inhibition of Migration
In *Mycobacterium paratuberculosis*-Infected Cattle
Treated With Rifabutin

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Pre-Treatment</th>
<th>Post-Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPD&lt;sup&gt;b&lt;/sup&gt;</td>
<td>$52.2 \pm 3.5^{a*}$</td>
<td>$60.1 \pm 7.2^*$</td>
</tr>
<tr>
<td>johnin</td>
<td>$43.3 \pm 3.9^*$</td>
<td>$52.7 \pm 7.9^*$</td>
</tr>
<tr>
<td>KLH</td>
<td>$14.2 \pm 1.8$</td>
<td>$11.5 \pm 6.6$</td>
</tr>
</tbody>
</table>

<sup>a</sup>Mean±SD.

<sup>b</sup>Derived from *M. bovis*.

<sup>c</sup>Keyhole limpet hemocyanin.

%Migration inhibition exceeded 98% with phytohemaglutinin in all assays.

*Indicates a positive response (>20% inhibition).
Figure 1. Ileal biopsy specimen taken before rifabutin treatment. There is a marked accumulation of macrophages in the lamina propria and submucosa. HE, 200X.
Figure 2. Section of ileum taken adjacent to the initial biopsy site after rifabutin treatment. Lesions noted are similar to the pre-treatment section. HE, 200X.
Figure 3. Lymph node biopsy specimen taken before rifabutin treatment. There is a moderate to marked accumulation of macrophages in the cortex. HE, 200X.
Figure 4. Mesenteric lymph node specimen taken adjacent to the initial biopsy site after rifabutin treatment. The section is similar to the pre-treatment biopsy sample.
LITERATURE CITED


CHAPTER 7

PRELIMINARY STUDIES WITH FERRITIN LABELING
OF BOINE BLOOD MONOCYTES INFECTED WITH MYCOBACTERIUM PARATUBERCULOSIS

Abstract

Bovine blood monocytes were pulse labeled with ferritin prior to infection with a field isolate of Mycobacterium paratuberculosis. Technical difficulty most likely related to fixation resulted in the absence of membranes in the cells examined. The cells did appear to phagocytize bacteria and take up the ferritin. Additional studies to accurately determine phagosome-lysosome fusion and killing ability of bovine blood monocytes is warranted.

Introduction

The intracellular fate of Mycobacterium paratuberculosis in cultured monocyte/macrophage systems has been previously examined. These studies have shown that monocytes and monocyte-derived macrophages are efficient in phagocytizing the organism but fail to kill it or prevent its replication. Inhibition of phagosome-
lysosome fusion is an important survival mechanism for several mycobacterial species.\textsuperscript{3,10,11,19}

Many of the mononuclear phagocytes seen in the granulomatous lesions of bovine paratuberculosis probably represent recently recruited blood monocytes instead of tissue macrophages.\textsuperscript{21} This is supported by the finding that mycobacterial granulomas have a rapid turn-over rate,\textsuperscript{1} probably resulting from the influx of blood monocytes instead of from division of resident macrophages.\textsuperscript{3} Blood monocytes may therefore play a considerable role in the kinetics and maintenance of the granulomatous lesions in bovine paratuberculosis. For this reason, phagosome-lysosome fusion in blood monocytes infected in vitro with \textit{Mycobacterium paratuberculosis} was qualitatively evaluated using ferritin to label lysosomes.

\textbf{Materials and Methods}

\textbf{Bacteria.}

A field isolate of \textit{Mycobacterium paratuberculosis} from a Brahman-cross bovine was cultured from feces using Herrold's egg-yolk media containing 2 \(\mu\)g/ml mycobactin J (Allied Laboratory, Ames, IA). Visible bacterial colonies were present after 6 months incubation. The bacteria was subcultured in Middlebrook's 7H9 broth supplemented with OADC (Difco
mycobactin J. The organism was grown for approximately 4 weeks, harvested by centrifugation, and washed with phosphate-buffered saline (PBS). The bacterial pellet was suspended in 15 ml of PBS and sonicated at 40% power for 30 seconds (Sonicator™ Model W-375, Ultrasonics, Inc.). The cell suspension was matched to a #4 McFarland standard equivalent to $12 \times 10^8$ bacteria/ml and 1 ml of cell suspension was added to 3 ml of RPMI 1640 containing 10% fetal bovine serum and 4 mM L-glutamine resulting in a final concentration of $3 \times 10^8$ bacteria/ml. Viability was assessed at >95% by fluorescein diacetate-ethidium bromide staining.\footnote{15}

**Preparation of Cells**

Heparinized venous blood (30 ml) from a healthy adult cow was diluted approximately in half with PBS and layered onto 1.080 g/ml Ficoll 400-Sodium diatrizoate (Histopaque, Sigma Chemical Co., St. Louis, Missouri). The blood and Ficoll gradient was centrifuged at 1000 X g for 45 minutes. The mononuclear cell layer removed and washed 3 times in PBS. Cell concentration was adjusted to $5 \times 10^6$ cells/ml in RPMI supplemented with 10% fetal bovine serum and 4mM L-glutamine. The cells were allowed to adhere in a 60 mm plastic petri dish for 1 hr. Non-adherent cells were washed off and the adherent cell
layer removed by scraping and adjusted to $1 \times 10^6$ cells in media. The cell suspension (3 ml) was allowed to re-adhere in a 60 mm petri dish for 1 hr before ferritin labeling.

**Ferritin Labeling**

A ferritin (equine spleen) solution (20 mg/ml) (Sigma Chemical Co., St. Louis, Missouri) was prepared in PBS containing 2% fetal bovine serum. The media from the adherent cells was removed and stored in a sterile container. The ferritin solution (4 ml) was added to the petri dish containing the cells and incubated at $37^\circ C$ for 3 hrs in the presence of 5% CO$_2$. The ferritin solution was removed and the cells washed 3 times with PBS. The original culture media was then returned to the petri dish and incubated an additional 3 hrs.

**Addition of Bacteria to Monocytes**

The bacterial suspension (2 ml), as described above, was added to the cells and incubated for 3 hrs. After incubation, non-phagocytized bacteria were removed by washing with PBS.

**Electron Microscopy**

The adherent cells were fixed to the petri dish with cold 3% glutaraldehyde in 0.1% sodium cacodylate buffer
(pH = 7.4) for one hour. The cells were then removed by scraping from the petri dish, washed twice in 0.1 M cacodylate buffer containing 5% sucrose, and gently pelleted at 200 X g. One drop of melted 4% agarose was added to the cell pellet and gently mixed. The agarose was allowed to harden and was minced into < 1mm cubes. Post fixation was performed with 1% OsO₄ in 0.1M cacodylate buffer (pH = 7.4) for one hour followed by dehydration in graded alcohols and propylene oxide. Infiltration and embedding was in Spurr's resin. Thin sections (60nm) were placed on uncoated copper grids and stained with uranyl acetate. The sections were viewed on a Zeiss EM-109 transmission electron microscope (Carl Zeiss Inc., Oberkochen, West Germany).

Results

Ferritin and bacteria were visible in all of the cells examined. No monocyte or bacteria-associated membranes were visible. The majority of the ferritin was present in round clumps, probably representing secondary lysosomes, and in vacuoles which also contained bacteria (Figures 1-4).

Discussion

Although ferritin labeling techniques for marking secondary lysosomes and morphologic evaluation of
phagosome-lysosome fusion has recently been criticized, d'Arcy Hart has provided a good defense for its use. The growth of intracellular parasites, such as Listeria monocytogenes, Legionella pneumophila, and Toxoplasma gondii is poor in monocytes, however, other studies indicated that blood monocytes and monocyte-derived macrophages supported the intracellular growth of Mycobacterium paratuberculosis, although macrophages by virtue of increased protein and lysozomal enzymes and increased phagocytic capacity are considered to be more effective than monocytes as killing cells in infectious granulomatous diseases.

The methods used to process and stain the cells in this study did not allow accurate visualization of cell membranes. Therefore the formation of a true peribacillary electron transparent zone and phagosome-lysosome membrane fusion was not be assessed. This zone has been seen in macrophages infected with a variety of mycobacteria including M. leprae, M. tuberculosis, M. lepraemurium, M. avium, and M. paratuberculosis. It has been interpreted that it has been suggested that some bacterial surface component is responsible for the formation of the electron transparent zone and that this zone may represent a defense mechanism for intracellular survival of the bacilli. Recently evidence indicates that this
zone is probably not produced by bacterial surface components because coating the organism with immune sera does not inhibit formation of the zone.

The kinetics and morphogenesis of mycobacterial granulomas appears variable, but mycobacterial granulomas in general are considered to have a rapid turnover rate. Development into epitheliod lesions may require 4 to 22 days depending upon the mycobacteria responsible for the lesion.\(^1\) High turnover granulomas have been described as being caused by non-toxic materials, and the macrophages contain relatively small amounts of the agent. This is the case in BCG and *M. tuberculosis* granulomas, but not in paratuberculosis. The lesions of paratuberculosis consist of epithelioid cells and giant cells which contain large numbers of bacteria. This is more indicative of low turnover granulomas.\(^1\)

It would be interesting to speculate that fresh blood monocytes are active in phagosome-lysosome fusion and killing of *M. paratuberculosis*. This would account for prevention of bacterial dissemination and localization of the infection to the intestine and regional lymph nodes. These hypotheses can not be tested adequately until better morphologic and cultural data is obtained.
Figure 1. The electron dense ferritin is present as round symmetrical clumps within the cell. (Uranyl acetate.)
Figure 2. The ferritin appears as fine grains in what may be a phagocytic vacuole. (Uranyl acetate).
Figure 3. A clump of ferritin adjacent to a structure resembling a bacterium. (Uranyl acetate)
LITERATURE CITED


CHAPTER 8

CONCLUSIONS AND SUMMARY

Cattle with chronic paratuberculosis had positive lymphocyte blastogenic (LB) and migration inhibition factor (MIF) assay results using johnin and \( M. \) \textit{bovis} PPD. This indicated that these cattle have the ability to respond immunologically to these related antigens. The LB and MIF assay responses with johnin in infected cattle were similar to non-infected cattle after sensitization with \( M. \) \textit{paratuberculosis} bacterin. This suggested that the in vitro response using these assays was of a normal magnitude. Infected cattle, in contrast to non-infected cattle, failed to develop positive skin tests and LB and MIF assay responses to keyhole limpet hemocyanin (KLH) after administration of a sensitizing dose of KLH in Freund's incomplete adjuvant. This lack of dermal response may have been related to the dose of KLH used or the minimum time allowed for a positive response to develop. Infected cattle apparently have the ability to respond immunologically to specific or cross-reactive antigen, yet fail to respond to a potent heterologous antigen. Failure of KLH responsiveness was not due to
inherent defects in mononuclear cell proliferation capacity as demonstrated by a positive response to mitogen-induced proliferation. Sensitization of infected cattle with homologous antigen did not alter LB and MIF assay responses detected before sensitization. This indicated an uninducible steady-state of reactivity to M. paratuberculosis antigens. Skin test reactivity to johnin and M. bovis PPD was significantly decreased by administration of M. paratuberculosis bacterin. It appeared that the cell type responsible for a positive skin test was made unreactive by high-dose antigen tolerance (desensitization).

Preliminary evidence suggested that phagosome-lysosome fusion in cultured blood monocytes may be assessed by pulse labeling with ferritin. The results obtained initially did not allow adequate interpretation, but suggested that phagosome-lysosome fusion had taken place. Additional studies using ferritin labeling appeared warranted.

Treatment of infected cattle with rifabutin significantly increased skin test reactivity to johnin. The reason for this may be that rifabutin damaged the bacilli and released previously hidden sensitizing antigens important in delayed hypersensitivity reactions. Damaged bacilli may have decreased their capacity to produce soluble substances which inhibit delayed
hypersensitivity reactions. It may also be speculated that rifabutin had a direct effect on the cells responsible for cutaneous hypersensitivity. Although no significant differences in interleukin-1 (IL-1) release, interleukin-2 (IL-2) production, and LB and MIF assays were noted after rifabutin treatment, trends suggested that these parameters were increasing. Beneficial results may be obtained by longer treatment duration and/or a different dosage regimen.

The production of IL-2 by peripheral blood mononuclear cells induced by concanavalin A was significantly suppressed in infected cattle. These cattle produced significant levels of IL-2 in response to johnin and *M. bovis* PPD which was unaltered after administration of sensitizing doses of these antigens. Interleukin-2 production in response to KLH could not be induced by sensitizing doses of KLH in Freund's incomplete adjuvant. Control cattle produced significantly greater amounts of IL-2 than infected cattle after sensitization. This indicated a defective IL-2 production mechanism in infected cattle. It can be speculated that this defect in IL-2 production results in inadequate numbers of *M. paratuberculosis*-reactive lymphocytes. Without reactive lymphocytes, killer cell and macrophage activation does not occur (discussed in Chapter 4). This may account for disease persistence in
paratuberculosis.

Augmentation of lymphocyte function in infected cattle by specific transfer factor to KLH and *M. paratuberculosis* resulted in positive MIF assay responses and increased IL-2 production to these antigens. The ability of transfer factor to increase IL-2 production in response to specific antigens has not been previously reported.

Blood monocytes from infected spontaneously released high levels of IL-1 in the absence of antigenic or mitogenic stimulus. There is no direct evidence that indicates that monocytes or macrophages in the lesions react similarly. The spontaneous release of IL-1 by blood monocytes probably results from a chronic state of activation of these cells induced by mycobacterial products. There may also be a defective IL-1 regulatory mechanism in infected cattle. Whether this IL-1 detected by in vitro assays is a functional lymphocyte activating monokine in vivo is not known. It could be speculated that some of the clinical signs seen in infected cattle, such as cachexia, muscle wasting, and neutrophilia, might be attributed to high systemic levels of IL-1.

On the basis of the information gathered in this study the principle immunologic defect in chronic bovine paratuberculosis appeared to be a decreased ability to
produce IL-2 and that this defect is at the level of the T-lymphocyte. A potential failure to regulate IL-1 release in blood monocytes was also discovered.
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