Polymerase Chain Reaction Analysis of TNF-α and IL-6 mRNA Levels in Whole Blood from Cattle Naturally or Experimentally Infected with *Mycobacterium paratuberculosis*

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ABSTRACT

Johne's disease is characterized by a chronic enteritis that results in granulomatous inflammation, cachexia, and eventual death of cattle infected with *Mycobacterium paratuberculosis*. The cytokines tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) have been associated with granuloma formation and wasting in other disease syndromes. The potential role of these cytokines in the development and progression of Johne's disease has not been investigated. Using the polymerase chain reaction (PCR) and specific bovine oligonucleotide cytokine primers and probes, we examined the expression of messenger RNA for these cytokines in whole blood from *M. paratuberculosis* infected and uninfected cattle. Cytokine mRNA levels were examined before and after in vitro incubation with *E.coli* lipopolysaccharide (LPS) and lipoarabinomannan (LAM) purified from *M. paratuberculosis*. Uninfected calves, experimentally infected calves, and naturally infected cattle all displayed similar cytokine mRNA expression patterns. However, individual animals demonstrated variability in the levels of IL-6 and TNF-α mRNA expression as determined by a semi-quantitative PCR method using ^{32}P-labelled oligonucleotide probes.

RÉSUMÉ

Les caractéristiques de la maladie de Johne chez les bovins infectés avec *Mycobacterium paratuberculosis* sont une inflammation de type granulomateuse de l'intestin et de la cachexie évoluant vers la mort. Les cytokines telles que le facteur alpha de nécrose tumorale (TNF-α) et l'interleukine-6 (IL-6) ont été associés avec la formation de granulomes et le dépérissement dans d'autres syndromes. Le rôle potentiel de ces cytokines dans le développement et la progression de la maladie de Johne n'a pas été étudié. Par réaction d'amplification en chaîne par la polymérase (ACP) à l'aide d'amorces et de sondes spécifiques à ces cytokines, l'expression de l'ARN messager de ces cytokines a été évaluée dans le sang entier de bovins infectés par *M. paratuberculosis* et des bovins non-infectés. Les niveaux d'ARNm ont été déterminés avant et après exposition in vitro au lipopolysaccharide (LPS) de *E.coli* et du lipoarabinomannane (LAM) purifié de *M. paratuberculosis*. Les patrons d'expression de l'ARNm des cytokines étaient similaires pour des veaux non-infectés, et des veaux infectés expérimentalement ou naturellement. Toutefois, des variations individuelles dans les niveaux d'expression de l'ARNm de IL-6 et TNF-α ont été observées par évaluation à l'aide d'une méthode d'ACP semi-quantitative utilisant des sondes d'oligonucléotides marquées avec du ^{32}P.

(Traduit par docteur Serge Messier)

INTRODUCTION

Johne's disease, a chronic granulomatous enteritis of ruminants caused by gastrointestinal infection with *Mycobacterium paratuberculosis*, is of major economic importance to the beef and dairy industries (1,2). Clinical signs of Johne's disease vary, and range from inapparent infection to intractable diarrhea and emaciation that ultimately lead to severe malnutrition, dehydration, and death (1,2). Although research into the pathogenesis of paratuberculosis has been limited, it is likely that at least some of the clinical signs associated with Johne's disease, such as granulomatous inflammation and chronic cachexia, result from the host response against *M. paratuberculosis* rather than from a direct effect of the organism.

The role of cytokines in the pathogenesis of clinical Johne's disease has not been addressed. Tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) are inflammatory cytokines that have been shown to have a wide range of biological activities (3,4). Previous research has demonstrated an important role for TNF-α in both granuloma formation and cachexia (4,5). Furthermore, *M. paratuberculosis* has also been implicated in Crohn's disease, an inflammatory bowel disease of humans that shares clinical and histopathological features with Johne's disease (1). TNF-α and IL-6 have been detected in plasma, circulating mononuclear cells, and intestinal tissue obtained from Crohn's disease patients (6–10), although their actual role in its pathogenesis remains unknown.

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Mycobacterium paratuberculosis and its cell wall components, muramyl dipeptide and lipoarabinomannan, are potent stimulators of bovine TNF-α and IL-6 in vitro (11). We were interested in determining whether blood from animals infected with *M. paratuberculosis* contains detectable mRNA for these inflammatory cytokines. A second purpose of this study was to determine whether healthy cattle, cattle naturally infected with *Mycobacterium paratuberculosis*, and calves experimentally infected with *Mycobacterium paratuberculosis*, differed in their ex vivo production of these cytokines. To do this, whole blood from cattle was analyzed by reverse transcriptase polymerase chain reaction for the accumulation of mRNA for TNF-α and IL-6 in response to bacterial lipopolysaccharide (LPS) and mycobacterial lipoarabinomannan (LAM).

MATERIALS AND METHODS

BLOOD COLLECTION

Venous blood was collected from the tail veins of healthy adult Holstein cattle using 0.38% sodium citrate as an anticoagulant as previously described (12). Blood was obtained in the same manner from experimentally and naturally infected cattle.

EXPERIMENTALLY INFECTED ANIMALS

As part of a larger experiment at the School of Veterinary Medicine, 16 female Holstein calves, 1 mo of age, were housed in conventional calf hutches on a local farm. Calves were infected at 1 mo of age during their evening feeding of milk on 3 consecutive days with either a high (10^8), or low (10^6), dose of *M. paratuberculosis* that had been harvested from the intestinal mucosa of a Holstein cow with clinical Johne's disease (13). Blood was collected as previously described 7 to 10 mo postinfection. Infected animals were shedding organisms during the first 2 mo postinfection.

NATURALLY INFECTED ANIMALS

Naturally infected adult Holstein cattle on several dairy farms in southern Wisconsin were identified as having mild clinical signs of Johne's disease. All cattle either had previously experienced, or were presently experiencing, protracted diarrhea. *Mycobacterium paratuberculosis* infection was verified serologically by agar gel immunodiffusion tests and by a commercial ELISA specific for *M. paratuberculosis* (14,15).

STIMULATION OF WHOLE BLOOD

Whole blood was aliquoted into 15 mL conical tubes (7 mL per tube). Samples from each donor were incubated with 10 μg per mL *Escherichia coli* lipopolysaccharide (Sigma Chemical, St. Louis, Missouri) or 10 μg per mL *Mycobacterium paratuberculosis* lipoarabinomannan (a generous gift from Dr. Ed Sugden, Agriculture Canada, Nepean, Ontario), for 1.5 h while being rotated at 39°C with 5% CO_2 (16). LAM preparations used in these studies were negative for LPS contamination by limulus amebocyte lysate testing.

ISOLATION OF TOTAL RNA

After stimulation, blood was centrifuged (1000 × *g* for 20 min at 22°C) and the buffy coat cells were collected. Buffy coat cells were immediately lysed with 500 μL ice cold guanidinium isothiocyanate (GIT). Samples were vortexed vigorously, 33 μL of 3 M sodium acetate (pH 5.2) was added, the mixture was vortexed again and 500 μL of H_2O-saturated phenol (Sigma) was added. The samples were then vortexed and 100 μL of chloroform was added. The samples were vortexed again, chilled on ice for 15 min, and then centrifuged in a microfuge (12 000 × *g* for 10 min at 22°C). The aqueous phase was removed to a new tube and mixed with an equal volume of phenol:chloroform:isoamyl alcohol (Sigma). The tubes were centrifuged (12 000 × *g* for 10 min at 22°C) and the aqueous phase transferred to a new tube. This was mixed with an equal volume of isopropanol and placed at −20°C for 90 min. The mixture was centrifuged (12 000 × *g* for 10 min at 22°C), the supernatant discarded, and the pellets washed once by resuspending with 750 μL ice cold 70% ethanol. After centrifugation (12 000 × *g* for 10 min at 22°C), the supernatant was discarded and the pellets allowed to air-dry. The pellets were then dissolved in 20 μL sterile H_2O and the yield of total RNA was determined spectrophotometrically (A_{260}/A_{280} ratios) using the following formula:

$$OD_{260} \times 43 \times 200 \times 10^{-3} = \mu g/\mu L$$
$$\text{Concentration} \times 20\ \mu L = \text{Yield}$$

PREPARATION OF cDNA

For each sample, 10 μg of total RNA was brought to a volume of 15 μL in a 500 μL microfuge tube using sterile H_2O. Samples were heated for 5 min at 65°C, cooled completely on ice, and 15 μL of reverse transcriptase mixture (2.0 μL Oligo d(T) [Promega Corp., Madison, Wisconsin, 1 mg per mL], 3.0 μL of dNTP mixture [Promega, 5 mM], 1.0 μL RNasin [Promega, 40 U/μL], 3.0 μL MMLV-RT [Gibco, 200 U/μL], and 6.0 μL of 5X RT buffer [Gibco BRL, Gaithersburg, MD]) was added. Samples were incubated at 42°C for 2 h and then frozen at −20°C until polymerase chain reactions were performed.

BOVINE CYTOKINE PRIMERS

Specific sequences for bovine cytokine primers and probes were obtained from the known cDNA sequences of bovine TNF-α (personal communication, William Wood, Genentech, San Francisco, California) and IL-6 (GenBank Accession #X57317). Primers and probes were constructed by the University of Wisconsin-Madison Biotechnology Center. 3' and 5' primer and probe sequences, and the size of the amplified products, were as follows : TNF-α (3'-CCA AAG TAG ACC TGC CCA GAC TC, 5'-ATG AGC ACC AAA AGC ATG ATC CGG, TNF-α probe 5'-TGG GGT CTC CCT GTG GCA AGG GCT CTT GAT, amplified fragment — 683 base pairs) and IL-6 (3'-CCA GCT ACT TCA TCC GAA TAG CTC, 5'-ATG AAC TCC CGC TTC ACA AGC GCC T, IL-6 probe 5'-TGC CAG TGT CTC CTT GCT GCT TTC ACA CTC, amplified fragment — 630 base pairs).

BOVINE OLIGONUCLEOTIDE PRIMER PURIFICATION

Lyophilized primers were dissolved in 400 μL of sterile H_2O and heated to 68°C for 5 min. 0.1 volumes (40 μL)

of 4 M ammonium acetate and 2.5 volumes (1 mL) of 95% ethanol were added to the primers and placed at −20°C for 1 h. The primers were then centrifuged (12 000 × *g* for 10 min at 22°C) and the pellets washed with 750 µL of 70% ethanol. After centrifugation (12 000 × *g* for 10 min at 22°C), the pellets were dried and then dissolved in 400 µL sterile H_2O. Samples were heated to 68°C and nucleic acid concentration was determined spectrophotometrically (A_{260}/A_{280} ratios) using the following formula :

(A_{260}) × (Dilution) × (30) × (10^{-3}) = µg/µL

(330) × (Primer Length) = grams/mole

[(µg/µL) / (grams/mole)] × (10^6) = µmoles primer

POLYMERASE CHAIN REACTION

For each sample the following reagents were added to a 500 µL microfuge tube: 34.25 µL sterile H_2O, 5.0 µL 10X Taq polymerase buffer (Promega), 2.0 µL dNTP mixture (Promega, 5 mM), 0.25 µL Taq polymerase (Promega, 5 U/µL), 2.5 µL MgCl (Promega, 25 mM), 2.5 µL 5' primer, 2.5 µL 3' primer (both primers diluted to 10 µM), and 1.0 µL of cDNA. The reaction mixtures were overlaid with 50 µL of mineral oil and amplified for 30 cycles (94°C, 30 s, 60°C, 1 min 20 s, 72°C, 2 min 5 s). Thirty cycles of amplification have previously been shown to fall within the linear portion of the amplification curve (data not shown). This was followed by 1 cycle at 72°C for 4 min. The PCR products were visualized by agarose gel electrophoresis. To do this, 8 µL of PCR reaction products were mixed with 2 µL 5X loading buffer and run on a 1.5% agarose gel with 1X tris acetate buffer. pGEM DNA size markers (Promega) were run as standards. The gel was stained with ethidium bromide and photographed. Negative controls were amplified in tubes without reverse transcriptase and no bands were observed after PCR amplification (data not shown).

5' END-LABELLING OF OLIGONUCLEOTIDE PROBES

TNF-α and IL-6 oligonucleotide probes were labelled using T4 polynucleotide kinase. Oligonucleotide probes were constructed across introns to eliminate the possibility that they might hybridize to genomic DNA sequences. The following reagents were added to each probe and incubated at 37°C for 30 min: 2.5 µL 10X T4 Kinase buffer (Promega), 17 µL ddH_2O, 1 µL bovine oligonucleotide probe (10 pmol), 3.5 µL [^{32}P]-ATP (Amersham, 6000 Ci/mmol), and 1 µL T4 polynucleotide kinase (Promega, 10 units). Reactions were stopped by the addition of 5 µL 0.5 M EDTA, pH 7.5. The labelled probes were recovered using Chroma-spin + TE columns (Clontech). These columns were loaded with the reaction mixtures and centrifuged at 700 × *g* for 5 min at 22°C. Labelled probe was collected and CPM were measured by liquid scintillation chromatography.

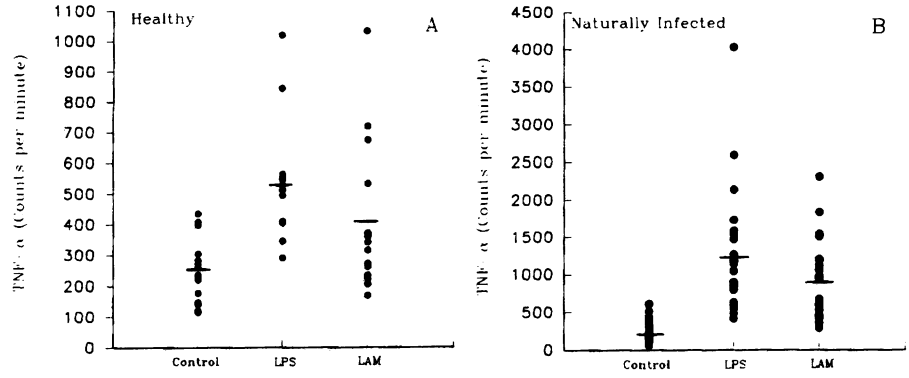

Figure 1. TNF-α mRNA expression in whole blood of (A) healthy cattle and (B) cattle naturally infected with *M. paratuberculosis* after in vitro incubation with medium (No Trt), 10 µg per mL *E.coli* lipopolysaccharide (LPS) or 10 µg per mL *M. paratuberculosis* lipoarabinomannan (LAM). Whole blood was rotated for 1.5 h at 39°C with 5% CO_2, buffy coat cells collected by centrifugation, and total RNA isolated. RNA was reverse transcribed and polymerase chain reaction amplification was performed. Reaction products were blotted to nitrocellulose, reacted with a ^{32}P-labelled probe specific for bovine TNF-α and CPM determined by AMBIS radioanalytic imaging. Each symbol represents an individual animal tested. Group means are denoted by horizontal lines. Group mean increases for LPS and LAM stimulated whole blood are statistically significant ($P < 0.05$) when compared to untreated whole blood (No Trt).

SLOT-BLOT HYBRIDIZATION

PCR reaction products (20–30 µL) were removed, diluted to 200 µL with TE buffer, and denatured by addition of 20 µL of 2 N NaOH and incubation at 65°C for 30 min. The samples were neutralized by the addition of 1 volume (220 µL) of 2 M ammonium acetate, and then applied with a slot-blot apparatus to nitrocellulose membranes that were presoaked in 1 M ammonium acetate. Following this, the nitrocellulose membranes were removed and baked for 1 h at 80°C in a vacuum oven. The membranes were then prehybridized for 1 h at 55°C 10 mL hybridization solution (6X SSC, 10 mM EDTA, pH 7.5, 2X Denhardt's Solution, 100 µg/mL sheared salmon sperm DNA[Sigma], and 1% SDS). After prehybridization, the appropriate oligonucleotide probe (4 × 10^6 CPM per mL of hybridization solution) was added and the membranes incubated for 18 h at 55°C. These oligonucleotide probes have been shown to detect their respective bovine TNF-α and IL-6 PCR products, and not to cross-react with each other (11). After hybridization, the membranes were washed with 2X SSC and 0.05% SDS with constant shaking first for 30 min at 22°C and then for 20 min with gentle agitation. Bound radioactivity was measured using the AMBIS Radioanalytic Imager and the results expressed as CPM.

STATISTICAL ANALYSIS

The data were analyzed for statistical significance by a one-way analysis of variance followed by a Bonferroni multiple comparisons test using the INSTAT software package (GraphPAD Software Inc., San Diego, California). The level of significance was set at $P < 0.05$.

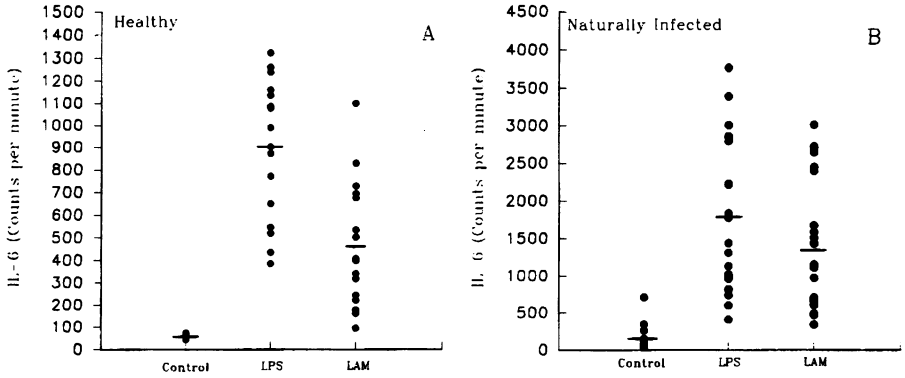

Figure 2. IL-6 mRNA expression in whole blood of (A) healthy cattle and (B) cattle naturally infected with *M. paratuberculosis*, after in vitro incubation with medium (No Trt), 10 μg per mL *E.coli* lipopolysaccharide (LPS) or 10 μg per mL *M. paratuberculosis* lipoarabinomannan (LAM). Whole blood was rotated for 1.5 h at 39°C with 5% CO_2, buffy coat cells collected by centrifugation, and total RNA isolated. RNA was reverse transcribed and polymerase chain reaction amplification was performed. Reaction products were blotted onto nitrocellulose and hybridized with ^{32}P-labelled probes specific for bovine IL-6. CPM were determined by AMBIS radioanalytic imaging. Each symbol represents an individual animal tested. Group means are denoted by horizontal lines. Group mean increases for LPS and LAM stimulated whole blood are statistically significant ($P < 0.05$) when compared to untreated whole blood (No Trt).

RESULTS

HEALTHY ANIMALS EXPRESS TNF-α AND IL-6 mRNA

Whole blood from uninfected donor cattle were incubated with 10 μg per mL *E.coli* lipopolysaccharide (LPS) or *Mycobacterium paratuberculosis* lipoarabinomannan (LAM) and analyzed for cytokine mRNA expression by polymerase chain reaction. Whole blood from healthy animals was characterized by spontaneous low levels of TNF-α mRNA expression (Figure 1A); however, IL-6 mRNA expression was not detected (Figure 2A). In contrast, expression of TNF-α and IL-6 mRNA was significantly increased ($P < 0.01$) over unstimulated whole blood levels after in vitro incubation with either LPS or LAM (Figures 1A and 2A).

NATURALLY AND EXPERIMENTALLY INFECTED CATTLE DISPLAY NORMAL CYTOKINE mRNA PATTERNS

Whole blood from naturally infected animals responded to incubation with LPS or LAM by increased ($P < 0.01$) mRNA expression for TNF-α and IL-6 (Figures 1B and 2B). Unstimulated blood from these animals showed background levels of mRNA expression that were similar to those obtained for whole blood from uninfected cattle (Figures 1B and 2B). Likewise, calves experimentally infected with *M. paratuberculosis* under controlled conditions did not express spontaneous levels of TNF-α or IL-6 mRNA above that of uninfected controls (Figures 3A and 3B). Whole blood from experimentally infected calves incubated with LAM in vitro demonstrated increased mRNA expression ($P < 0.01$) as compared with untreated whole blood (Figures 3A and 3B). No significant differences were observed in mRNA expression between groups of calves that were inoculated with 10^6 or 10^8 *M. paratuberculosis* (Figure 3).

DISCUSSION

The results of this study demonstrate the TNF-α and IL-6 mRNA expression patterns of whole blood from cattle infected with *Mycobacterium paratuberculosis* as detected with the polymerase chain reaction and specific bovine oligonucleotide probes. Semi-quantitative PCR was utilized in this study to determine the presence or absence of the cytokine being examined. Due to the limitations of semi-quantitative PCR, no comparisons of actual amounts of cytokine mRNA were made between healthy, naturally-infected, or experimentally-infected cattle, although similar mRNA expression patterns are noted.

Healthy cattle did not express significant spontaneous levels of TNF-α or IL-6 mRNA in their blood. In vitro incubation with *E.coli* LPS or *M. paratuberculosis* LAM caused a significant increase in whole blood levels of TNF-α and IL-6 mRNA. Similar cytokine mRNA expression patterns were observed with whole blood from cattle that were naturally infected or experimentally infected with *M. paratuberculosis*.

This is the first report of cytokine mRNA expression patterns in cattle naturally and experimentally infected with *M. paratuberculosis*. Infected cattle, at the specific time points that we tested them, do not constitutively express TNF-α or IL-6 mRNA in their peripheral blood. Johne's disease is a chronic infection whose course spans years from the time of initial infection to obvious clinical disease. We examined the experimentally infected animals relatively early (7 to 10 mo) after inoculation with *M. paratuberculosis*. Therefore, these animals were not experiencing clinical signs of Johne's disease. In contrast, the naturally infected animals were ELISA and AGID positive and demonstrated mild clinical signs of Johne's disease, including protracted diarrhea. The absence of cytokine expression in the peripheral blood of cattle infected with clinical Johne's disease demonstrates that *M. paratuberculosis* and its cell wall components do not stimulate persistent release of cytokines by blood cells. In addition, peripheral blood mononuclear cells from *M. paratuberculosis*-infected cattle were not primed for the rapid and enhanced release of cytokines in response to mycobacterial cell wall components (LAM) or a non-specific stimulus (LPS) in vitro. These studies do not rule out the possibility that cytokines may be expressed in the peripheral blood at earlier or later stages of Johne's disease. Alternatively, at different stages of infection, the immune response to *M. paratuberculosis* may be compartmentalized and, therefore, only detectable at the local sites of infection in the intestinal tract.

Similar studies have analyzed the mRNA expression patterns of inflammatory cytokines in human inflammatory bowel diseases. McCabe et al demonstrated that intestinal mucosa

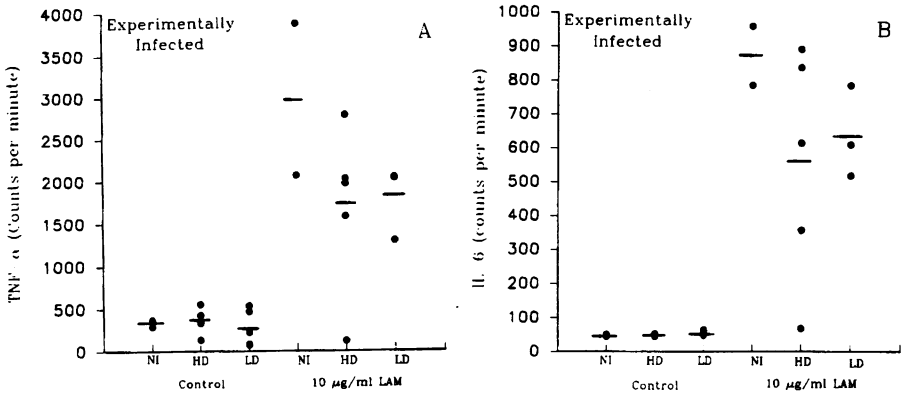

Figure 3. TNF-α (A) and IL-6 (B) mRNA expression in whole blood of cattle experimentally infected (NI, not infected, HD, 10^8 organisms, LD, 10^6 organisms) with *M. paratuberculosis*, after in vitro incubation with medium or 10 μg per mL *M. paratuberculosis* lipoarabinomannan (LAM). Whole blood was rotated for 1.5 hours at 39°C with 5% CO_2, buffy coat cells collected by centrifugation, and total RNA isolated. RNA was reverse transcribed and polymerase chain reaction amplification was performed. Reaction products were blotted onto nitrocellulose and hybridized with ^{32}P-labelled probes specific for bovine TNF-α and bovine IL-6. CPM were determined by AMBIS radioanalytic imaging. Each symbol represents an individual animal tested. Group means are denoted by horizontal lines. Group mean increases for LAM stimulated whole blood are statistically significant ($P < 0.05$) when compared to untreated whole blood (No Trt).

and lamina propria mononuclear cells from ulcerative colitis (UC) and Crohn's disease (CD) patients express elevated levels of IL-1β and TGF-β mRNA as compared with healthy controls (17). In addition, IL-1, IL-4, IL-5, and IL-6 steady state mRNA expression was above that of control patients. Isaacs et al examined mucosal biopsies from patients with UC or CD for cytokine mRNA expression (18). They found significantly increased IL-1β and IL-6 mRNA expression in UC and CD patients as compared with healthy controls. Cappello et al also demonstrated elevated mRNA expression of IL-1β and TNF-α in UC and CD patients using in situ hybridization techniques (19). In contrast to analyzing mRNA expression in the intestine, other studies have examined the production of inflammatory cytokines in peripheral blood. Satsangi et al (10) and Mahida et al (8) have shown that peripheral blood mononuclear cells isolated from Crohn's disease patients spontaneously release IL-1. After in vitro stimulation with LPS, these cells produced larger amounts of IL-1 than did LPS-stimulated cells from healthy donors. TNF-α has also been detected in the serum of children suffering from UC and CD (9). Other studies have demonstrated elevated levels of IL-6 in the sera of UC and Crohn's disease patients (6,7,20). We did not examine mRNA expression in mononuclear cells and, therefore, were unable to conclude whether the serum cytokines detected were produced in the periphery or at local sites of infection in the intestinal tract. It is interesting that these changes in cytokine expression have been noted in human inflammatory bowel disease, yet we noted no difference in TNF-α and IL-6 expression between normal cattle and cattle with Johne's disease.

In summary, this study provides new information regarding the production of inflammatory cytokines in the pathogenesis of Johne's disease. Similar strategies and tools could be used to examine intestinal tissues from healthy and infected cattle for the expression of inflammatory cytokines during clinical Johne's disease.

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REFERENCES

1. **CHIODINI RJ.** Crohn's disease and the mycobacterioses: A review and comparison of two disease entities. Clin Microbiol Rev 1989; 2: 90–117.
2. **CHIODINI RC, VAN KRUININGEN HJ, MERKAL RS.** Ruminant paratuberculosis (Johne's disease): The current status and future prospects. Cornell Vet 1984; 74: 218–262.
3. **VAN SNICK J.** Interleukin-6: An overview. Ann Rev Immunol 1990; 8: 252–278.
4. **LE J, VILCEK J.** Biology of disease. Tumor necrosis factor and interleukin 1: Cytokines with multiple overlapping biological activities. Lab Invest 1987; 56: 234–248.
5. **KINDLER V, SAPPINO AP, GRAU GE, PIGUET PF, VASSALLI P.** The inducing role of tumor necrosis factor in the development of bactericidal granulomas during BCG infection. Cell 1989; 56: 731–740.
6. **GROSS V, ANDUS T, CAESAR I, ROTH M, SCHOLMERICH J.** Evidence for continuous stimulation of interleukin 6 production in Crohn's disease. Gastroenterology 1992; 102: 514–519.
7. **MAHIDA YR, KURLAC L, GALLAGHER A, HAWKEY CJ.** High circulating concentrations of interleukin-6 in active Crohn's disease but not in ulcerative colitis. Gut 1991; 32: 1531–1534.
8. **MAHIDA YR, WU KC, JEWELL DP.** Enhanced production of interleukin-1β by mononuclear cells isolated from mucosa with active ulcerative colitis or Crohn's disease. Gut 1989; 30: 835–838.
9. **MURCH SH, LAMKIN VA, SAVAGE MO, WALKER-SMITH JA, MACDONALD TT.** Serum concentrations of tumour necrosis factor-α in childhood chronic inflammatory bowel disease. Gut 1991; 32: 913–917.
10. **SATSANGI J, WOLSTENCROFT RA, CASON J, AINLEY CC, DUMONDE DC, THOMPSON RPH.** Interleukin 1 in Crohn's disease. Clin Exp Immunol 1987; 67: 594–605.
11. **ADAMS JL, CZUPRYNSKI CJ.** Mycobacterial cell wall components induce the production of TNF-α, IL-1, and IL-6 by bovine monocytes and the murine macrophage cell line RAW 264.7. Microb Path 1994; 16: 401–411.
12. **ZURBRICK BG, FOLLETT DM, CZUPRYNSKI CJ.** Cytokine regulation of the intracellular growth of *Mycobacterium paratuberculosis* in bovine monocytes. Infect Immun 1988; 56: 1692–1697.
13. **RATNAMOHAN TN, SPENCER TL.** A technique for the purification of *Mycobacterium paratuberculosis* from the ileal mucosa of infected cattle. Aust Vet J 1986; 63: 185–187.
14. **COX JC, DRANE DP, JONES SL, RIDGE S, MILNER AR.** Development and evaluation of a rapid absorbed enzyme immunoassay test for the diagnosis of Johne's disease in cattle. Aust Vet J 1991; 68: 157–160.
15. **SOCKETT DC, CONRAD TA, THOMAS CB, COLLINS MT.** Evaluation of

four serological tests for bovine paratuberculosis. J Clin Microbiol 1992; 30: 1134–1139.
16. **SUGDEN EA, SAMAGH BS, BUNDLE DR, DUNCAN JR.** Lipoarabinomannan and lipid-free arabinomannan antigens of *Mycobacterium paratuberculosis*. Infect Immun 1987; 55: 762–770.
17. **MCCABE RP, SECRIST H, BOTNEY M, EGAN M, PETERS MG.** Cytokine mRNA expression in intestine from normal and inflammatory bowel disease patients. Clin Immunol Immunopathol 1993; 66: 52–58.
18. **ISAACS KL, SARTOR RB, HASKILL S.** Cytokine messenger RNA profiles in inflammatory bowel disease mucosa detected by polymerase chain reaction amplification. Gastroenterology 1992; 103: 1587–1595.
19. **CAPPELLO M, KESHAV S, PRINCE C, JEWELL DP, GORDON S.** Detection of mRNAs for macrophage products in inflammatory bowel disease by in situ hybridization. Gut 1992; 33: 1214–1219.
20. **HYAMS JS, FITZGERALD JE, TREEM WR, WYZGA N, KREUTZER DL.** Relationship of functional and antigenic interleukin-6 to disease activity in inflammatory bowel disease. Gastroenterology 1993; 104: 1285–1292.