Expression of molecular markers in blood of neonatal foals with sepsis

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Objective—To determine gene expression of selected molecular markers (tumor necrosis factor [TNF]-α, interleukin [IL]-1β, IL-6, IL-8, IL-10, procalcitonin [PCT], and transforming growth factor [TGF]-β) in the blood of healthy and sick foals.

Animals—28 sick foals without sepsis, 21 foals with sepsis, and 21 healthy foals.

Procedures—Total RNA was extracted from blood samples and converted into complementary DNA (cDNA). Gene expression was measured for the molecular markers by use of real-time PCR assay, and final quantitation was performed with the comparative threshold cycle method.

Results—Samples from all foals yielded transcription for all markers. Expression of TNF-α and TGF-β was significantly lower and that of IL-8 significantly greater in the sick-nonseptic and septic groups, compared with the healthy group. No significant difference in expression of IL-1β, IL-6, and PCT was found between the healthy group and the 2 sick groups. Expression of IL-10 was significantly greater in nonsurvivors, compared with survivors.

Conclusions and Clinical Relevance—The cytokine profile in foals with sepsis may suggest an immunosuppressive state. Expression of IL-10 may be a marker for identification of foals with a guarded prognosis. (*Am J Vet Res* 2006;67:1045–1049)

Bacterial infection continues to be a major cause of morbidity and death in neonatal foals.[1-3] Neonatal foals are often subjected to extensive diagnostic evaluation and empirical systemic antimicrobial treatment because the prognosis for sepsis largely depends on early identification and treatment. The definitive diagnosis of septicemia requires positive results of bacteriologic culture of blood, which requires a minimum of 48 to 72 hours, yields a positive result in only 12% to 80% of cases, and may not always be available.[3] A previous study[2] has examined the laboratory findings associated with sepsis. There is, however, a lack of consensus on the essential tests that would identify neonates with acute infection. Attempts to identify neonatal foals with septicemia have included development of a sepsis scoring system that combines historical information and objective and subjective data to derive a numerical representation of the foal's condition.[4,5] The sensitivity and specificity of this scoring system were reported to be 93% and 86%, respectively. However, estimation of the sensitivity and specificity may be seriously flawed because scoring systems strive to incorporate large amounts of clinically relevant data into a single representative numerical value. Although this may be beneficial in concentrating one's attention on the most important components of the clinical situation, it also results in loss of information. Another limitation arises from the fact that the pathophysiologic mechanisms in neonatal foals with systemic inflammatory response syndrome are consistent, regardless of the primary etiology.[3] In recent years, various investigators have evaluated some highly sensitive and specific inflammatory markers used to diagnose neonatal sepsis.[6-8] Although these markers are highly sensitive and specific, they require sophisticated kits and their analysis is time-consuming. Gene expression of inflammatory mediators in the blood of human neonates has been investigated in recent years. Expression of mRNA in umbilical blood cells indicates that cytokine production is present in neonates and has been used as a surrogate marker for sepsis and bacterial virulence.[9,10] To our knowledge, measurement of cytokine gene transcription has never been reported in neonatal foals with sepsis. We hypothesized that gene expression of selected biological markers may be altered in foals with sepsis. The purpose of the study reported here was to determine gene expression of selected molecular markers (TNF-α, IL-1β, IL-6, IL-8, IL-10, PCT, and TGF-β) in the blood of healthy and sick foals by use of real-time PCR assays.

Abbreviations

TNF	Tumor necrosis factor
IL	Interleukin
PCT	Procalcitonin
TGF	Transforming growth factor
VMTH	Veterinary Medical Teaching Hospital
cDNA	Complementary DNA
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
C_T	Comparative threshold cycle

Materials and Methods

Case selection—Forty-nine client-owned foals evaluated at the VMTH at the University of California, Davis, during the 2005 foaling season represented the case material for this study. All foals were < 5 days of age. Using historical, physical, and clinicopathologic data, a sepsis score was calculated for each foal. On the basis of sepsis score and results of bacteriologic culture of blood, each foal was assigned to 1 of 2 patient groups: a sick-nonseptic group (sepsis score < 11 and negative results of bacteriologic culture of blood; 28 foals) or a septic group (sepsis score ≥ 11 with or without positive

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Table 1—Relative* expression (mean ± SD) of selected biological markers in the blood of healthy foals, sick-nonseptic foals, and septic foals.

Group	TNF-α	IL-1β	IL-6	IL-8	IL-10	PCT	TGF-β
Healthy	0.74 ± 2.19	−1.06 ± 16.77	1.05 ± 2.14	1.44 ± 1.14	0.52 ± 2.17	2.34 ± 3.81	1.24 ± 1.69
Sick-nonseptic	−2.79 ± 4.44	1.37 ± 2.08	−0.35 ± 3.52	6.72 ± 12.69	−0.04 ± 3.20	1.06 ± 1.69	−3.96 ± 5.42
Septic	−3.28 ± 2.98	0.83 ± 6.34	−1.80 ± 4.42	59.25 ± 92.73	2.28 ± 4.20	20.64 ± 46.03	−7.84 ± 10.22

*Relative to a calibrator value.

results of bacteriologic culture of blood or sepsis score < 11 with positive results of bacteriologic culture of blood; 21 foals). The sick-nonseptic group was composed of foals with hypoxic encephalopathy, partial failure of passive transfer, meconium impaction, angular limb deformity, trauma, or bladder rupture. The 21 septic foals had a sepsis score ranging from 10 to 18 (mean ± SD, 13.7 ± 1.9). The following pathogens were cultured from 9 of the 21 (43%) foals: *Escherichia coli* (n = 3), *Actinobacillus* spp (2), *Klebsiella pneumoniae* (1), *Enterococcus faecium* (1), *Stenotrophomonas* spp (1), and *Streptococcus viridans* (1). Treatment performed by referring veterinarians on the sick foals prior to referral to the VMTH consisted of administration of antimicrobials (16 sick-nonseptic foals; 7 septic foals), nonsteroidal anti-inflammatory drugs (7; 1), dextrose-containing crystalloid fluids (8; 5), or equine plasma (3; 2). An additional 21 foals < 5 days of age served as healthy controls. These foals were born at the Center for Equine Health, University of California, Davis, and were considered healthy on the basis of normal results of physical examination and clinicopathologic findings within reference ranges.

Blood samples—Blood was collected from each foal by use of special RNA tubes[a] at the time of initial evaluation. The tubes are used to collect 3 mL of blood and have been developed for stabilization and isolation of total RNA from blood.

RNA extraction, cDNA synthesis, and quantitation of cytokine transcripts—Total RNA was extracted from the RNA tubes by use of a nucleic acid preparation station[b] according to the manufacturer's recommendations. Thereafter, 20 µL of each freshly extracted nucleic acid sample was digested with DNase[c] for 15 minutes at 37°C to remove genomic DNA; DNase was then inactivated at 95°C for 5 minutes. Before cDNA synthesis, 1 µL of each sample was tested for genomic DNA background by use of the equine housekeeping gene GAPDH as a marker. Only samples with negative results for GAPDH were processed for cDNA synthesis, whereas samples with positive results were redigested and retested. The cDNA from each sample was synthesized by use of 200 units of reverse transcriptase[d] in a 40-µL final volume (pH, 8.3) containing 50mM Tris-HCl, 50mM KCl, 8mM $MgCl_2$, 0.5mM dNTPs, 40 units of RNase, 0.5mM dithiothreitol, and 600 ng of random hexadeoxyribonucleotide (pd[N]6) primers.[e] The reaction was performed at 50°C for 120 minutes. After inactivation at 95°C for 5 minutes, the reaction volume was adjusted to 100 µL with nuclease-free water. The cDNA samples were stored at –20°C until analysis.

Real-time PCR systems for equine GAPDH, TNF-α, IL-1β, IL-6, IL-8, IL-10, PCT, and TGF-β were as described.[11,12] The PCR reactions contained 400nM of each primer, 80nM of the probe and mastermix,[f] and 5 µL of the diluted cDNA sample in a final volume of 12 µL. The samples were amplified in a combined thermocycler-fluorometer[g] for 2 minutes at 50°C, 10 minutes at 95°C, and 40 cycles of 15 seconds at 95°C and 60 seconds at 60°C.

Final quantitation was performed by use of the C_T method.[13] First, the C_T for the target amplicon and for the internal control (GAPDH) was determined for each sample.

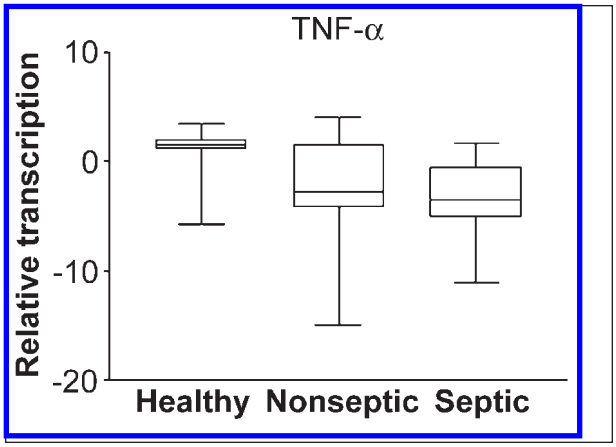

Figure 1—Box plots of relative* expression of TNF-α in blood from healthy, sick-nonseptic, and septic foals. Box represents the 25th to 75th percentile, horizontal line represents the median, whiskers represent the range. *Relative to a calibrator value.

Differences in the C_T for the target and the internal control, called ΔC_T, were calculated to normalize for differences in the amount of total nucleic acid added to each reaction and the efficiency of the reverse transcriptase step. The ΔC_T for each sample from the sick-nonseptic and septic foals was subtracted from the ΔC_T of the calibrator (healthy foals). This difference was called the $\Delta\Delta C_T$. Finally, the amount of target, normalized to an internal control and relative to the calibrator, was calculated by $2^{-\Delta\Delta CT}$. Thus, results are expressed as an n-fold difference relative to the calibrator.

The Mann-Whitney *U* test was used to determine significant ($P < 0.05$) differences in cytokine gene expression among the various foal groups.

Results

All foals had certain levels of transcription for TNF-α, IL-1β, IL-6, IL-8, IL-10, PCT, and TGF-β (**Table 1**). Expression of TNF-α was significantly ($P < 0.001$) lower in the sick-nonseptic and septic groups, compared with the healthy group (**Figure 1**), whereas no significant difference was found between the 2 patient groups. Expression of IL-8 was significantly greater in the sick-nonseptic ($P = 0.016$) and septic ($P = 0.018$) group, compared with the healthy group (**Figure 2**). No significant difference was found between the 2 patient groups for IL-8 expression. For IL-10, no significant difference in expression was detected between sick-nonseptic foals and septic foals ($P = 0.09$; **Figure 3**) or between healthy and patient groups. Similar to TNF-α, TGF-β expression was significantly ($P < 0.001$) lower in the sick-nonseptic and septic groups, compared with the healthy group (**Figure 4**), whereas no significant difference was found between the 2 patient groups. No significant difference in gene expression of IL-1β, IL-6, and PCT was found between the healthy and patient groups.

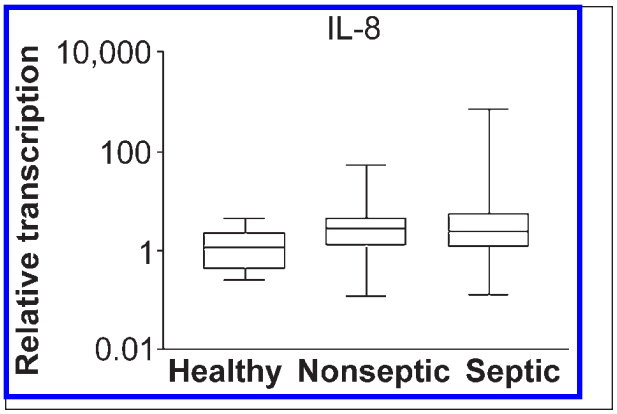

Figure 2—Box plots of relative expression of IL-8 in blood from healthy, sick-nonseptic, and septic foals. *See* Figure 1 for key.

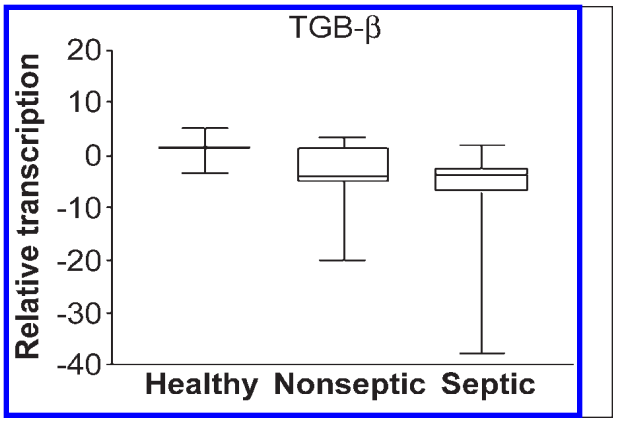

Figure 4—Box plots of relative expression of TGF-β in blood from healthy, sick-nonseptic, and septic foals. *See* Figure 1 for key.

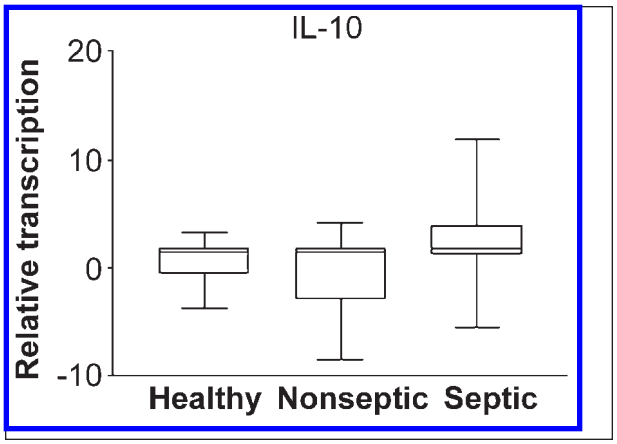

Figure 3—Box plots of relative expression of IL-10 in blood from healthy, sick-nonseptic, and septic foals. *See* Figure 1 for key.

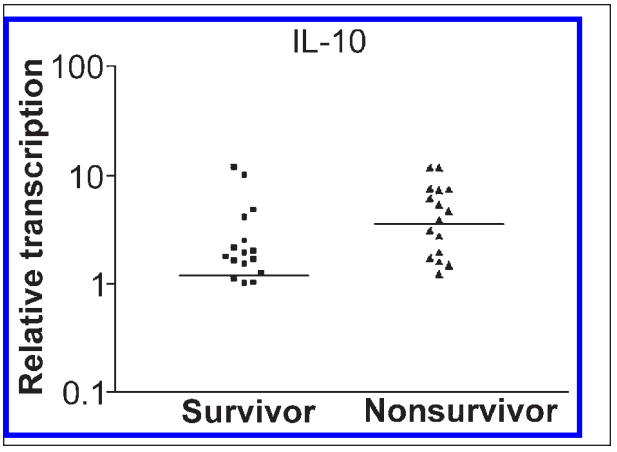

Figure 5—Scatterplots of relative expression of IL-10 in blood from surviving and nonsurviving foals. Horizontal bars indicate mean values. *See* Figure 1 for key.

Table 2—Relative* expression (mean ± SD) of selected biological markers in the blood of surviving and non-surviving foals.

Group	TNF-α	IL-1β	IL-6	IL-8	IL-10	PCT	TGF-β
Survivors	1.02 ± 2.72	0.52 ± 2.33	0.88 ± 2.50	1.92 ± 3.71	1.65 ± 1.47	–0.20 ± 5.77	0.63 ± 4.17
Nonsurvivors	0.57 ± 2.25	–1.08 ± 5.72	0.37 ± 5.48	12.82 ± 41.68	35.69 ± 94.64	3.56 ± 5.73	–2.95 ± 10.91

*Relative to a calibrator value

To evaluate the prognostic value of selected biological markers, cytokine profiles of foals discharged from the hospital (n = 26 [20 sick-nonseptic and 6 septic foals]) were compared with foals that died or were euthanized because of treatment failure (19 [5 sick-nonseptic and 14 septic foals]). Four foals euthanized because of financial constraints were not included in this evaluation. Expression of IL-10 was significantly ($P = 0.008$) greater in the nonsurvival group, compared with the survival group (**Figure 5**). No significant difference in expression of TNF-α, IL-1β, IL-6, IL-8, PCT, and TGF-β was found between survivors and nonsurvivors (**Table 2**).

Discussion

Neonatal sepsis and its sequelae are among the leading causes of death in foals. Although neonatal foals often develop a systemic inflammatory response syndrome with detrimental effects, the effects in older foals and adults tend to be more organ-restricted. There is growing evidence that this process is crucially mediated by the action of distinct inflammatory cytokines.[14] The inflammatory response to infection is a highly conserved and regulated reaction. Proinflammatory cytokines that activate cellular defenses are produced, followed by production of anti-inflammatory cytokines to attenuate and control the proinflammatory response. This anti-inflammatory response, referred as compensatory anti-inflammatory response syndrome, limits the effects of proinflammatory cytokines. If balance cannot be established and homeostasis is not restored, a proinflammatory state with severe organ dysfunction will ensue.[15] Evaluation of gene expression of biological markers has become a novel approach to investigate the complex mechanism of neonatal infection and has diagnostic and prognostic value in human neonatal sepsis.[9,10] In the present study,

all foals had levels of transcription for selected markers of proinflammation (TNF-α, IL-1β, and IL-6), chemotaxis (IL-8), bacterial infection (PCT), and anti-inflammation (TGF-β and IL-10). With the exception of IL-10, expression of the biological markers did not differ between sick-nonseptic and septic foals. The large SD of the values of some molecular markers (ie, IL-1β, IL-8, PCT, and TGF-β), mainly septic foals, may have been related to the degree of sepsis in those foals. The role of anti-inflammatory drugs and biological products administered to some foals prior to their referral to the VMTH may have had an influence on the transcription of inflammatory cytokines; however, because of the small number of foals that received such treatment, this issue could not be addressed. The significantly lower expression of proinflammatory cytokine TNF-α and growth factor TGF-β and the greater expression of chemokine IL-8 in both patient groups may have indicated dysregulation in inflammatory pathways in the sick foals.

Tumor necrosis factor-α is produced predominantly by $CD4^+$ T cells and macrophages as well as by neutrophils and has multiple proinflammatory actions. Early in sepsis, proinflammatory reactions induced by TNF-α dominate. In contrast, expression of TNF-α decreases rapidly in the later stages of sepsis.[16] This may explain why foals in the 2 patient groups in the present study had low expression of TNF-α despite an underlying inflammatory disease process. The lower expression of TNF-α observed in the 2 patient groups may have been mediated by the anti-inflammatory cytokine IL-10. Results of some studies[17-19] suggest that the inhibitory effect of IL-10 on macrophage proinflammatory cytokines, such as TNF-α, occurs at the level of gene expression because of decreased transcription and at the level of protein expression.

Interleukin-8 is primarily produced by macrophages and endothelial cells. Interleukin-8 is considered an inflammatory mediator and a chemotactic agent for neutrophils, and its expression occurs secondary to tissue injury or inflammation.[20] In a recent study,[9] high gene expression of IL-8 in umbilical cord blood of human neonates was found to be a sensitive marker for neonatal infection. Interleukin-8 was the only cytokine with greater expression in sick-nonseptic and septic foals, compared with healthy foals. However, no significant difference was detected between the 2 patient groups. It is possible that larger groups are necessary to differentiate sick-nonseptic foals from septic foals via IL-8 gene expression in blood.

Procalcitonin was first described as a sepsis-induced protein in the early 1990s.[21] Results of numerous clinical studies[22-24] indicate that PCT is primarily induced during severe systemic inflammation caused by bacterial infections but not during other types of inflammation. There are conflicting data concerning WBCs as a source of PCT in sepsis.[25,26] The present study revealed no significant difference in gene expression of PCT among the 3 foal groups, which is in agreement with a recent study[27] that revealed low expression of PCT in the WBCs of septic animals and humans.

Transforming growth factor-β is regarded as a cytokine that may downregulate production of acute-phase reactants and the inflammatory cytokine cascade in sepsis.[28,29] Two studies[30,31] of TGF-β mRNA concentrations in blood from septic human patients revealed that TGF-β expression was lower than that of healthy subjects. This is in agreement with the present study, which revealed that sick foals had decreased TGF-β expression, compared with healthy foals. The exact mechanisms by which TGF-β is downregulated in septic patients and its pathophysiologic role in sepsis are not clear. They may be related to the ability of TGF-β to modulate macrophage activity by rendering monocytes hyporesponsive.

Interleukin-10 is a pleiotropic cytokine produced by activated subpopulations of T cells, B cells, monocytes-macrophages, and keratinocytes.[32] It appears to be a potent regulator of immune function that inhibits the production of cytokines, including TNF-α, IL-1, IL-6, and interferon-γ in monocytes.[33] In addition, IL-10 can suppress macrophage, T-cell, and natural killer cell effector functions.[32] Several studies[34-36] reveal that IL-10 inhibits the immune responsiveness of antigen-presenting cells, such as monocytes, macrophages and dendritic cells. It also inhibits lymphocyte and phagocytic functions, which are essential for an adequate immune response to invading microbes. In particular, an experimental study[33] revealed that IL-10 inhibits the production of proinflammatory cytokines, such as TNF-α, IL-1, and IL-6, by activated macrophages, which is in agreement with results of the present study. Significantly greater IL-10 expression was detected in nonsurviving foals, compared with surviving foals. Clinical data suggest that the increased expression of IL-10 in injured human patients appears to be an indicator of poor prognosis and higher risk for infection, multiple organ failure, and death.[30,34,37] Further studies are needed to assess the immunologic status of septic equine neonates to develop better diagnostic and treatment modalities.

a. Tempus blood RNA tube, Applied Biosystems, Foster City, Calif.
b. ABI Prism 6100 Nuclei Acid PrepStation, Applied Biosystems, Foster City, Calif.
c. DNase, Promega, Madison, Wis.
d. SuperScript III, Invitrogen, Carlsbad, Calif.
e. Random hexamers, Invitrogen, Carlsbad, Calif.
f. TaqMan Universal PCR Mastermix, Applied Biosystems, Foster City, Calif.
g. ABI Prism 7700 Sequence Detection System, Applied Biosystems, Foster City, Calif.

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