

Comparative analysis of cytokine gene expression in cerebrospinal fluid of horses without neurologic signs or with selected neurologic disorders

Nicola Pusterla, DVM; W. David Wilson, BVMS, MS; Patricia A. Conrad, DVM, PhD; Samantha Mapes, MS; Christian M. Leutenegger, DVM, PhD

Objective—To determine gene transcription for cytokines in nucleated cells in CSF of horses without neurologic signs or with cervical stenotic myelopathy (CSM), West Nile virus (WNV) encephalitis, equine protozoal myeloencephalitis (EPM), or spinal cord trauma.

Animals—41 horses (no neurologic signs [$n = 12$], CSM [8], WNV encephalitis [9], EPM [6], and spinal cord trauma [6]).

Procedures—Total RNA was extracted from nucleated cells and converted into cDNA. Gene expression was measured by use of real-time PCR assay and final quantitation via the comparative threshold cycle method.

Results—Cytokine genes expressed by nucleated cells of horses without neurologic signs comprised a balance between proinflammatory tumor necrosis factor- α (TNF- α), anti-inflammatory cytokines (interleukin [IL]-10 and transforming growth factor [TGF]- β), and Th1 mediators (interferon [IFN]- γ). Cells of horses with CSM mainly expressed genes for TNF- α , TGF- β , and IL-10. Cells of horses with WNV encephalitis mainly expressed genes for IL-6 and TGF- β . Cells of horses with EPM mainly had expression of genes for IL-6, IL-8, IL-10, TNF- α , IFN- γ , and TGF- β . Cells from horses with spinal cord trauma had expression mainly for IL-6; IFN- γ ; TGF- β ; and less frequently, IL-2, IL-10, and TNF- α . Interleukin-8 gene expression was only detected in CSF of horses with infectious diseases.

Conclusions and Clinical Relevance—Despite the small number of CSF samples for each group, results suggest distinct gene signatures expressed by nucleated cells in the CSF of horses without neurologic signs versus horses with inflammatory or traumatic neurologic disorders. (*Am J Vet Res* 2006;67:1433–1437)

ABBREVIATIONS

CSM	Cervical stenotic myelopathy
WNV	West Nile virus
EPM	Equine protozoal myeloencephalitis
NSAID	Nonsteroidal anti-inflammatory drug
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
TNF	Tumor necrosis factor
IFN	Interferon
IL	Interleukin
iNOS	Inducible nitric oxide synthase
TGF	Transforming growth factor

analysis of cytologic specimens, protein concentration, and appearance and composition of CSF helps determine involvement of the CNS, and specific changes in the CSF are well correlated with some infectious diseases; however, this is not without limitations for diagnosis of certain neurologic diseases. The CSF values may be within reference ranges in a horse with severe neurologic deficits because the lesion is extradural, collection occurred early or late in the course of disease, or the CSF collection site is too far away from the lesion to allow changes to be detected. Additionally, different inflammatory neurologic diseases have similar changes in the CSF, making differentiation solely based on CSF analysis impossible. Detection and quantitation of biological markers (eg, cytokines, chemokines, and other effector molecules) in the neural tissue of humans have facilitated definitive diagnosis by allowing clinicians to better differentiate between immunologic, infectious, oncologic, and traumatic neurologic disorders.¹⁻⁷ The immunoregulatory mechanisms ensuing from neurologic tissue injury or inflammation also take place in the CSF compartment itself, and CSF cells are involved in the production of inflammatory as well as immunosuppressive cytokines.⁸⁻¹⁴ The objective of this study was to determine the transcriptional activity for cytokines, chemokines, and other effector molecules in nucleated cells of the CSF collected from horses without neurologic signs and from horses with selected neurologic disorders

Materials and Methods

Horses—Forty-one horses were selected for this study. Twelve horses did not have neurologic signs and were euthanized because of acute musculoskeletal injuries. These horses had normal results of neurologic examination prior to euthanasia, unremarkable results of CSF evaluation, and no

Cerebrospinal fluid analysis is indicated whenever disease of the CNS is suspected. Routine

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From the Departments of Medicine and Epidemiology (Pusterla, Leutenegger, Mapes, Wilson) and Pathology, Microbiology, and Immunology (Conrad), and the Lucy Whittier Molecular and Diagnostic Core Facility (Mapes, Leutenegger), School of Veterinary Medicine, University of California, Davis, CA 95616.

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Address correspondence to Dr. Pusterla.

histologic abnormalities on routine cut sections of brain or spinal cord. Eight horses with neurologic abnormalities had CSM (on the basis of survey radiographs) and normal results of CSF evaluation and histologic examination. Nine horses with neurologic signs had histopathologic findings consistent with WNV encephalitis. Results of CSF analysis in these horses were within reference ranges in 2 horses and indicated lymphocytic pleocytosis in 7, whereas protein concentration was greater than reference range in only 1 horse. Six horses had EPM on the basis of histologic lesions in neural tissues and immunohistochemical detection of *Sarcocystis neurona* parasites. Results of CSF fluid evaluation were within reference ranges in all horses with EPM. Six horses with acute onset of recumbency had cervical cord trauma detected at necropsy. Results of CSF evaluations of these horses were unremarkable. Horses from each group with neurologic signs had similar severity in neurologic signs and histopathologic changes.

CSF collection—Cerebrospinal fluid was collected from the atlantooccipital space in all horses. Collection of CSF from horses without neurologic signs, horses with WNV encephalitis, and horses with EPM was performed immediately after euthanasia by use of IV barbiturate overdose. The interval between onset of clinical signs and CSF collection was 1 to 10 days (mean, 2.6 days) and 5 to 14 days (mean, 11.5 days) for horses with WNV encephalitis and EPM, respectively. Seven of 9 horses with WNV encephalitis were being treated with NSAIDs^a (mean duration, 1.6 days) at the time of CSF collection. Although 3 horses with EPM had not received any medication at the time of CSF collection, the remaining 3 horses were receiving NSAIDs^a (mean duration, 4.3 days) and antiprotozoal medication^b (mean duration, 7.9 days) at the time the CSF samples were collected. Collection of CSF from horses with CSM and spinal cord trauma was performed during general anesthesia after radiographic evaluation of the spinal cord. The interval between onset of clinical signs and CSF collection was 2 weeks to 6 months (mean, 2.2 months) for horses with CSM and 3 to 12 days (mean, 5.5 days) for horses with spinal cord trauma. Although no horse with CSM was receiving any treatment at the time of CSF collection, the 6 horses with spinal cord trauma had all received NSAIDs^a prior to CSF collection (mean duration, 4.2 days). All CSF samples were collected in glass tubes without any coagulant and kept on ice until further processing.

RNA extraction, cDNA synthesis, and quantitation of cytokine transcripts—After CSF collection, 5 to 10 mL of each sample was centrifuged and the cell pellet was suspended in lysis buffer prior to extraction of nucleic acids by use of a commercial kit.^c Thereafter, the nucleic acid was digested with RNase-free DNase I^d at 37°C for 15 minutes, and the reaction was completed with 5 minutes at 95°C, followed by chilling on ice. Presence of undigested genomic DNA was tested 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. Reverse transcription reactions were performed with 20 µL of total RNA in a 40-µL final volume containing 600 ng of random hexadeoxyribonucleotide (pd[N]6) primers,^e 50mM Tris-HCl (pH, 8.3), 50mM KCl, 8mM MgCl₂, 0.5mM dNTPs, 40 units RNase inhibitor,^f 0.5mM dithiothreitol, and 50 units of RNA H-reverse transcriptase^g 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 was analyzed immediately or stored at -20°C until use.

Real-time PCR systems for equine GAPDH, TNF- α , IFN- γ , IL-2, IL-6, IL-8, IL-10 and TGF- β were used as

described.^{15,16} The PCR reactions contained 400nM of each primer, 80nM of the probe and master mix,^h and 5µL of the diluted cDNA sample in a final volume of 12 µL. The samples were amplified in a combined thermocycler-fluorometerⁱ for 2 minutes at 50°C, 10 minutes at 95°C, and then 40 cycles of 15 seconds at 95°C and 60 seconds at 60°C.

Final quantitation was performed via the comparative threshold cycle method¹⁷ and reported as relative transcription or the n-fold difference relative to the cDNA calibrator. Mean gene transcription from nucleated cells of the CSF from horses without neurologic signs was chosen as the calibrator. For cytokines not expressed in the CSF of horses without neurologic signs, the group with the lowest mean gene transcription was used as the calibrator. The Mann-Whitney test was used to determine significant ($P < 0.05$) differences in cytokine gene expression among groups.

Results

Results of CSF analysis from horses without neurologic signs and horses with selected neurologic disorders were tabulated (Table 1). The housekeeping gene GAPDH was expressed in all samples, which indicated successful RNA extraction and cDNA transcription. The level of GAPDH expression was similar among the 5 groups ($P > 0.05$), indicating that there were no differences in efficiency of RNA extraction or level of cDNA transcription. Nucleated cells collected from the CSF of horses without neurologic signs had gene expression for IL-10, TNF- α , IFN- γ , and TGF- β in most samples; for IL-2 and IL-6 in half of the samples; and for iNOS in only 1 sample. Interleukin-8 transcripts were not detected in any sample (Table 2). Nucleated cells in the CSF of horses with CSM had gene expression mainly for TNF- α , whereas IL-2, IL-10, and TGF- β gene expression was detected in half of the samples. Interleukin-6 and IFN- γ gene expression was rare, and IL-8 and iNOS gene expression was not detected in any sample. In contrast, cytokine gene profiles from the nucleated cells collected from the CSF of horses with WNV encephalitis were characterized by gene expression for IL-6 and TGF- β in most samples and for IL-8, IL-10, TNF- α , and IFN- γ in half of the samples. Interleukin-2 gene expression was rarely detected, and iNOS transcription was not detected in any of the samples. Nucleated cells from horses with EPM often expressed IL-6, IL-8, IL-10, TNF- α , IFN- γ , and TGF- α . Gene expression for IL-2 and iNOS was not detected in any sample. Gene expression for IL-6, IL-10, IFN- γ , and TGF- β was detected in approximately half of the CSF samples collected from horses with spinal cord trauma. In the same horses, IL-2 and TNF- β gene expression was rarely detected and no IL-8 or iNOS gene expression was detected.

Significant differences were detected in IL-6 gene expression (Figure 1) among the following groups: horses with WNV encephalitis versus horses without neurologic signs ($P = 0.003$), horses with WNV encephalitis versus horses with EPM ($P = 0.001$), horses with spinal cord trauma versus horses without neurologic signs ($P = 0.03$), and horses with spinal cord trauma versus horses with EPM ($P = 0.01$). For TNF- α gene expression (Figure 2), significant differences were found between samples from horses without neurologic signs and with either WNV encephalitis ($P =$

Table 1—Results (mean ± SD [range]) of CSF analysis in horses without neurologic signs and horses with selected neurologic disorders.

Group (No. horses)	RBCs (cells/ μ L)	Total nucleated cells (cells/ μ L)	CSF protein (mg/dL)	Interpretation (No. of horses)
No neurologic signs (12)	(2.4 ± 4.4) 1–17	(1.8 ± 0.8) 1–3	(43.2 ± 12.6) 26–59	RR (12)
CSM (8)	(5.4 ± 8.2) 1–20	(2.5 ± 2.5) 1–6	(47.8 ± 13.9) 28–66	RR (8)
WNV encephalitis (9)	(163.3 ± 178.7) 1–420	(39.8 ± 30.8) 1–97	(82.6 ± 51.6) 44–192	RR (2) Lymphocytic pleocytosis (7)
EPM (6)	(34.5 ± 62.6) 1–170	(3.2 ± 2.7) 1–6	(57.3 ± 19.2) 50–85	RR (6)
Spinal cord trauma (6)	(64.5 ± 89.8) 1–128	(2.0 ± 1.5) 1–3	(73 ± 24.9) 46–95	RR (6)

Mean ± SD (range).
RR = Within reference range.¹⁸

Table 2—Relative gene transcription (mean ± SD) for selected biological markers in CSF nucleated cells from horses without neurologic signs and horses with selected neurologic disorders.

Group	IL-2	IL-6	IL-8	IL-10	TNF- α	IFN- γ	iNOS	TGF- β
No neurologic signs (12)	1.0 ± 0.9	1.2 ± 1.0	NA	0.9 ± 0.5	1.7 ± 2.3	1.7 ± 1.7	1.0	0.7 ± 0.7
CSM (8)	3.0 ± 2.6	3.9	NA	0.4 ± 0.1	8.6 ± 14.1	4.2 ± 0.1	NA	1.0 ± 1.3
WNV encephalitis (9)	0.8 ± 0.1	407.6 ± 777.9	1.0 ± 0.3	1.2 ± 1.0	4.1 ± 3.7	2.0 ± 2.9	NA	5.1 ± 6.3
EPM (6)	NA	0.3 ± 0.3	1.4 ± 0.3	1.5 ± 0.3	10.5 ± 6.4	2.0 ± 0.7	NA	4.7 ± 2.4
Spinal cord trauma (6)	2.5 ± 0.1	59.4 ± 41.2	NA	0.9 ± 3.5	7.1 ± 0.8	2.8 ± 2.8	NA	3.4 ± 4.1

NA = Not applicable.

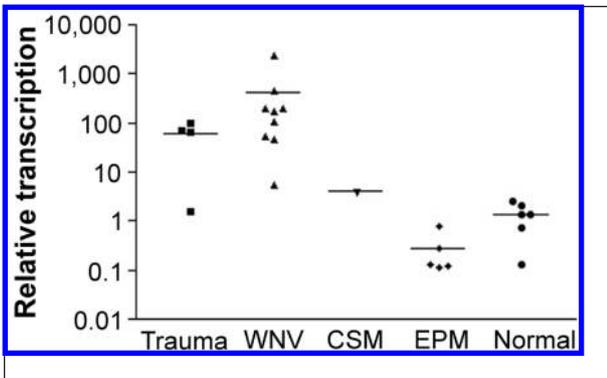


Figure 1—Scatterplots of relative gene expression for IL-6 in CSF nucleated cells from horses without neurologic signs and horses with selected neurologic diseases. Horizontal bars indicate mean values. Normal = Horses without neurologic signs.

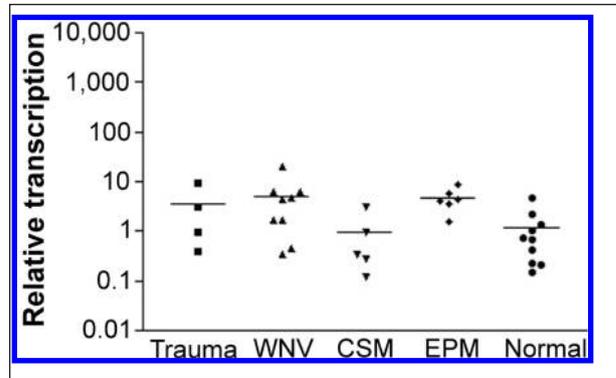


Figure 3—Scatterplots of relative gene expression for TGF- β in CSF nucleated cells from horses without neurologic signs and horses with selected neurologic diseases. See Figure 1 for key.

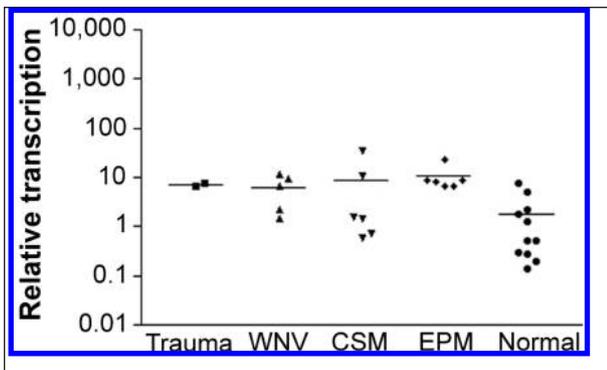


Figure 2—Scatterplots of relative gene expression for TNF- α in CSF nucleated cells from horses without neurologic signs and horses with selected neurologic diseases. See Figure 1 for key.

0.03) or EPM ($P = 0.002$). Significant differences were also found for TGF- β gene expression (Figure 3) among the following groups: horses without neurologic signs and with either WNV encephalitis ($P = 0.03$)

or EPM ($P = 0.004$), as well as horses with CSM and either WNV encephalitis ($P = 0.04$) or EPM ($P = 0.009$). No significant differences in gene expression for IL-2, IL-8, IL-10, IFN- γ , and iNOS were found among the groups in which gene expression for these cytokines were detected.

Discussion

Studies^{9,11,13} investigating the pathophysiologic mechanisms of neurologic inflammatory diseases in humans reveal that immunoregulatory mechanisms take place not only in neural tissues, but also in the CSF compartment itself and that CSF cells are involved in gene expression and production of proinflammatory as well as immunosuppressive cytokines. Data from the present study indicate that immunomodulatory mechanisms take place in the CSF compartment of horses, regulated by CSF cells in different but specific ways depending on the etiology of the condition. Although IL-6 and TNF- α seem to promote inflammation in the CSF of horses with inflammatory or infectious diseases, TGF- β conversely seems to be involved in down-

regulation of the inflammatory activity. The study reported here was based on the detection of transcriptional activity of selected biological markers and not on their protein concentrations because most protein assays are not available for horses and their detection limit may be greater than concentrations found in CSF. In addition, evidence reveals that cytokines pass across the blood-brain barrier, thereby confusing interpretation of cytokines in CSF.^{13,19-21} Furthermore, a recent study²² reveals that mRNA gene transcription and production of cytokines measured in neural tissues are in phase with each other.

Results of the present study indicated different disease-associated patterns of cytokine gene expression in nucleated cells from CSF. The gene expression was normalized against GAPDH, a cellular message for which expression is relatively invariant; therefore, it is unlikely that the increase in cytokine gene expression in the CSF of certain groups of horses with neurologic signs resulted solely from increased cellularity, but instead reflected the activation status of nucleated cells in the CSF. Changes in cell population (ie, predominance of small mononuclear cells), as seen for horses with WNV encephalitis, may also account for variations in cytokine gene profiles. The cytokine gene profiles expressed by nucleated cells from the CSF of horses without neurologic signs corresponded to a balance between proinflammatory mediators (TNF- α), anti-inflammatory mediators (IL-10 and TGF- β), and Th1 cytokines (IFN- γ) in most of the samples. Genes for other markers of inflammation (IL-2 and IL-6) were less commonly expressed. Lack of gene expression for chemotactic IL-8 in the CSF of clinically normal horses, horses with CSM, and those with spinal cord trauma was not surprising because its detection is commonly associated with development of CNS diseases caused by various infections.²³⁻²⁵ Inducible nitric oxide synthase gene expression was detected in only 1 sample; that sample was collected from a horse without neurologic signs. Inducible nitric oxide synthase is a transcriptionally regulated enzyme that synthesizes nitric oxide from L-arginine and has a key role in the pathophysiologic mechanisms of systemic inflammation and sepsis. Animal model studies reveal that at baseline there is no detectable iNOS gene expression in the brain and that early in the course of systemic inflammation there is a profound induction of iNOS mRNA in vascular, glial, and neuronal structures of the brain; however, no induction has been reported in nucleated cells from the CSF, which is in agreement with our results.²⁶

Although cytokines play a major role in different noninflammatory conditions, we did not detect a significant increase or decrease in cytokine mRNA expression between horses with CSM and horses without neurologic signs. Horses with CSM mainly had expression for proinflammatory (TNF- α) and anti-inflammatory (TGF- β) cytokines and rarely for IL-6 and IFN- γ , compared with horses without neurologic signs. It is possible that the reported variations in the number of horses with expression for specific mediators for each noninflammatory group may have simply reflected the small number of horses assigned to each group. In regard to CSM, we conclude that there does

not appear to be a specific cytokine gene expression pattern in nucleated cells that would allow differentiation from other neurologic diseases. Wallerian degeneration in the central and peripheral nervous systems consists of degradation and phagocytosis of axons and their myelin sheath distal to the site of injury by macrophages. A recent study²⁷ reveals increased mRNA expression of 3 proinflammatory markers (monocyte chemoattractant protein-1, macrophage inflammatory protein-1, and IL-1) in peripheral nerves, but not in the spinal cord undergoing Wallerian degeneration. These specific markers of phagocytosis have not yet been evaluated in horses. Although purely speculative, it is possible that CSF collected distal to the lesion (ie, via lumbosacral puncture) may have a different cytokine gene pattern than CSF collected from the atlantooccipital space of horses with CSM.

Viral, bacterial, and protozoal neurologic diseases induce proinflammatory and anti-inflammatory cytokine expression in neural tissues by blood-derived infiltrating monocytes.^{10-12,28-30} Tumor necrosis factor- α and IL-6 especially seem to initiate and promote inflammation in the CNS, whereas TGF- β has antagonistic effects and suppresses inflammation in the subarachnoidal space. All horses with WNV encephalitis in the present study had expression for IL-6 and TGF- β that was significantly greater than that in horses without neurologic signs. Five of those horses also had significantly greater gene expression for TNF- α than that of horses without neurologic signs. Our results were in agreement with those of studies^{8,12,29,30} that evaluated gene transcription of cytokines in the CSF of humans and dogs affected with viral pathogens. All samples from horses with EPM had expression for TNF- α and TGF- β that was significantly greater than that in horses without neurologic signs, which was in agreement with results of a prior study¹ that evaluated gene expression of selected cytokines in neural tissues of horses affected by EPM. The difference in gene expression for inflammatory cytokines between horses with WNV encephalitis and horses with EPM may have been related to different stages of an ongoing inflammatory response. The IL-8 transcripts were only observed in samples from horses with WNV encephalitis or EPM. Interleukin-8 is produced mainly by macrophages as a response to various infectious pathogens.²³⁻²⁵ Therefore, IL-8 may represent an important marker for differentiating between noninfectious and infectious diseases in horses. Future investigation will be necessary to confirm whether the amount of gene expression for these mediators correlates with the clinical state of the horse and is of prognostic relevance in neurologic horses.

Spinal cord trauma is characterized by tissue injury and inflammation that results in marked production of proinflammatory cytokines and chemotactic mediators that lead to accumulation of neutrophils.³¹ The cytokine gene expression profile of horses with spinal cord trauma was fairly similar to the profile of horses without neurologic signs, with the exception of significantly increased IL-6 gene expression. Production of proinflammatory cytokines, including TNF- α and IL-6, at the site of injury regulates the precise cellular events

after spinal cord injury. A recent study³² reveals that retrograde cell death in supra spinal regions that are distant from the core of spinal cord injury occurs via signaling processes that involve activation of proinflammatory cytokine genes and the intracellular caspase-3 pathway. The high gene expression for IL-6 observed in nucleated cells in CSF collected from the atlantooccipital space of half of the horses with spinal cord trauma in the present study may indicate existence of the same pathway. It would have been interesting to compare the cytokine gene expression patterns in CSF samples collected from the atlantooccipital and lumbosacral spaces in these horses.

- a. Flunixin meglumine, Schering-Plough Corp, Union, NJ.
- b. Ponazuril, Bayer Corp, Shawnee Mission, Kan.
- c. DNeasy tissue kit, Qiagen, Valencia, Calif.
- d. Promega, Madison, Wis.
- e. Random hexamers, Invitrogen, Carlsbad, Calif.
- f. Invitrogen, Carlsbad, Calif.
- g. SuperScript III, Invitrogen, Carlsbad, Calif.
- h. TaqMan Universal PCR Mastermix, Applied Biosystems, Foster City, Calif.
- i. ABI PRISM 7700 sequence detection system, Applied Biosystems, Foster City, Calif.
- j. Pusterla N, Leutenegger CM, Conrad PA, et al. Cytokine gene signatures in neural tissue of horses diagnosed with EPM or EHV-1 myeloencephalopathy (abstr), in *Proceedings. 23rd Annu Meet Am Coll Vet Intern Med Forum* 2005;427.

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