Reduced Levels of Nitric Oxide Metabolites in Cerebrospinal Fluid Are Associated with Equine Protozoal Myeloencephalitis

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Received 23 July 2001/Returned for modification 3 October 2001/Accepted 7 January 2002

Equine protozoal myeloencephalitis (EPM) is a disease of horses that is primarily associated with infection with the apicomplexan Sarcozystis neurona. Infection with this parasite alone is not sufficient to induce the disease, and the mechanism of neuropathogenesis associated with EPM has not been reported. Nitric oxide (NO) functions as a neurotransmitter, a vasodilator, and an immune effector and is produced in response to several parasitic protozoa. The purpose of this work was to determine if the concentration of NO metabolites (NOx) in the cerebrospinal fluid (CSF) is correlated with the development of EPM. CSF NOx levels were measured before and after transport-stressed, acclimated, or dexamethasone-treated horses (n = 3 per group) were experimentally infected with S. neurona sporocysts. CSF NOx levels were also compared between horses that were diagnosed with EPM after natural infection with S. neurona and horses that did not have clinical signs of disease or that showed no evidence of infection with the parasite (n = 108). Among the experimentally infected animals, the mean CSF NOx levels of the transport-stressed group, which had the most severe clinical signs, was reduced after infection, while these values were found to increase after infection in the remaining groups that had less severe signs of EPM. Under natural conditions, horses with EPM (n = 65) had a lower mean CSF NOx concentration than clinically normal horses with antibodies (Abs) against S. neurona (n = 15) in CSF, and horses that developed ataxia (n = 81) had a significantly lower mean CSF NOx concentration than horses that did not have neurologic signs (n = 24). In conclusion, lower CSF NOx levels were associated with clinical EPM, suggesting that measurement of CSF NOx levels could improve the accuracy of diagnostic tests that are based upon detection of S. neurona-specific Abs in CSF alone and that reduced NO levels could be causatively related to the development of EPM.

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Equine protozoal myeloencephalitis (EPM) is a disease of horses that is primarily attributed to the onset of ataxia after infection with the protozoan parasite Sarcozystis neurona (8). The disease affects the central nervous system (CNS); and clinical signs may be representative of those affected by any area of the CNS including stumbling, depression, ataxia, spasticity, weakness, and cranial nerve deficits that are sometimes accompanied by associated muscle atrophy (9, 12, 21). Progression of clinical signs is variable. EPM is reportedly the most prevalent cause of neurologic disease of horses in the Americas, and the estimated annual cost of diagnosis and treatment of EPM in the United States has been estimated at $110 million (9). Over 30% of the horses in some parts of the United States have antibodies (Abs) to S. neurona (4, 5, 29). However, it has been estimated that ≤1% of exposed horses develop EPM (i.e., ataxia in association with S. neurona-specific Abs in the cerebrospinal fluid [CSF]), indicating that additional factors also contribute to the onset of this disease (9). An accurate experimental model for EPM has not been developed, under scoring the complex etiology of this disease and the need for further understanding of the pathogenic mechanisms responsible for its onset. Investigation of aspects of EPM other than the infection alone will help elucidate its poorly understood pathogenesis.

Previously, we reported on the association between stress and EPM and how this has been used to develop an experimental equine model for EPM (31). Three groups of horses were experimentally infected with S. neurona sporocysts from feral opossums either immediately upon arrival as a test of the effect of transport stress, after a 2-week acclimation period, or after dexamethasone treatment as a test of the effect of immunosuppression. All of these horses seroconverted and developed anti-S. neurona Ab titers in their CSF after sporocyst inoculation. Interestingly, the transport-stressed group had horses with the highest clinical scores but the fewest animals with histopathologic lesions in the CNS, while the dexamethasone-treated group had horses with lower clinical scores but a larger number of individual horses with lesions in the CNS (31). These results indicate that the severity of the clinical signs due to EPM was not associated with the severity of lesions in the CNS but, rather, was due at least in part to a factor other than parasite load.

Nitric oxide (NO) is a versatile molecule that can function as a neurotransmitter, a vasodilator, and an immune effector, indicating that EPM could be associated with altered levels of NO production in association with S. neurona infection of the CNS. All three NO synthase (NOS) isoforms, inducible NOS, endothelial NOS, and neuronal NOS, have been reported in the mammalian CNS (7). Evidence of either increased or reduced levels of NO production has been reported.
in association with several neurologic disorders (1, 16, 19, 26), and NO production has also been demonstrated in the presence of gamma interferon (IFN-γ) and parasitic protozoa (14, 20, 23, 27, 33, 34). Increased iNOS activity has also been reported in the CNS and CSF of rabbits with experimental bacterial meningitis, and mercaptoethylguanidine, a peroxynitrite scavenger and iNOS inhibitor, was found to reduce the pathogenesis of this disease without affecting the bacterial infection level (16). The purpose of this study was to investigate the association between NO production and the onset of EPM.

The approach to this investigation was to compare the NO metabolites nitrite (NO−2) and nitrate (NO−3), collectively referred to as NO−x, in the CSF of horses with experimental or naturally occurring cases of EPM. CSF was collected from experimental horses before and after infection with S. neurona sporocysts collected from feral opossums. Challenge groups included horses subjected to transport-related stress, acclimated horses, or horses treated with dexamethasone prior to infection. NO−x levels were also measured in the CSF collected from horses admitted to The Ohio State University Veterinary Teaching Hospital (OSU VTH) for clinical neurologic examination. Lower CSF NO−x levels were associated with the onset of clinical EPM, suggesting novel approaches to the diagnosis and control of this disease.

**MATERIALS AND METHODS**

**Horses.** For experimental infection, nine horses that were seronegative for S. neurona were randomly assigned to three groups (a transport-stressed group, an acclimated group, and a group treated with dexamethasone after acclimation) prior to inoculation with S. neurona sporocysts from feral opossums, which is reported elsewhere (31). Briefly, these horses were shipped to the site of study and subjected to neurologic examinations by a masked observer on the day of arrival, at the time of inoculation, and biweekly thereafter. Transport-stressed horses were inoculated with S. neurona within a few hours upon arrival (day 0). Acclimated horses were inoculated with S. neurona 14 days after arrival. Steroid-treated horses received 0.5 mg of dexamethasone per kg of body weight (intramuscularly) on days 12 to 14 prior to inoculation with S. neurona on day 14, and semiweekly dexamethasone doses (0.2 mg/kg) were continued for the remainder of the study. CSF samples were collected on the days of infection and necropsy, as described previously (13).

For the groups in the natural exposure study, CSF samples were collected only once from each of the equine patients admitted to the OSU VTH from December 1997 through June 1998 for neurologic examination. Each horse was subjected to two independent neurologic examinations by different clinical observers prior to collection of CSF. Other potential causes of neurologic disease in horses were randomly assigned to three groups (a transport-stressed group, an acclimated horses, or horses treated with dexamethasone prior to inoculation with S. neurona, sporocysts from feral opossums, which is reported elsewhere (31). Briefly, these horses were shipped to the site of study and subjected to neurologic examinations by a masked observer on the day of arrival, at the time of inoculation, and biweekly thereafter. Transport-stressed horses were inoculated with S. neurona within a few hours upon arrival (day 0). Acclimated horses were inoculated with S. neurona 14 days after arrival. Steroid-treated horses received 0.5 mg of dexamethasone per kg of body weight (intramuscularly) on days 12 to 14 prior to inoculation with S. neurona on day 14, and semiweekly dexamethasone doses (0.2 mg/kg) were continued for the remainder of the study. CSF samples were collected on the days of infection and necropsy, as described previously (13).

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**RESULTS**

**CSF NO−x levels in experimentally infected horses.** NO−x levels were measured in CSF collected prior to and following experimental infection of horses to determine if NO production could explain the differences in clinical EPM observed between stressed, acclimated, and dexamethasone-treated horses (31). No difference in CSF NO−x levels was observed among the acclimated, dexamethasone-treated, or transport-stressed groups before they were exposed to S. neurona; but NO−x levels were different (P < 0.05) among each of these groups after infection (Fig. 1). CSF NO−x concentrations increased for the acclimated and dexamethasone-treated groups after exposure to S. neurona. Conversely, the CSF NO−x concentration of the transport-stressed animals, which had the highest ataxia scores, decreased after the onset of EPM. **CSF NO−x levels in naturally infected horses.** The CSF NO−x concentrations in experimentally infected horses indicated that lower CSF NO−x levels could be associated with EPM, but not all of these samples were collected at the time of peak clinical disease and the small numbers of individuals in these groups made quantitative differences in clinical signs
difficult to demonstrate. Thus, 105 CSF samples collected from horses admitted to the OSU VTH were assayed to determine if lower NO\(_x\) concentrations could be associated with EPM in animals with cases that developed under natural conditions. These animals were arranged into four groups on the basis of the presence (C+) or absence (C-) of anti-\(S.\ neurona\) Abs in CSF in combination with ataxia scores of 0 (normal) or 1 (ataxic). Only those horses that were both C+ and A+ were considered to have clinical EPM.

The C+ A+ group (i.e., horses with EPM) had a lower mean CSF NO\(_x\) concentration than the C+ A- group by use of the mixed model (\(P = 0.012\)), and this difference also approached significance in the multiple pairwise comparison by use of the more conservative Tukey-Kramer adjustment (\(P = 0.0571\)) (Fig. 2). The mean CSF NO\(_x\) level for the C+ A+ group was also lower than that for the C- A- group, but this difference was not significant (\(P = 0.0920\)), possibly due to the small number of horses in the latter group (\(n = 9\)). No significant difference was observed between the C+ A+ group and the C- A+ group. The A+ horses had a lower mean CSF NO\(_x\) concentration than A- horses (\(P = 0.0068\)), indicating that lower CSF NO\(_x\) levels could be associated with neurologic disease in general rather than EPM specifically. A total of 80.25% of the A+ horses were also C+, but this value was not significantly different from the 62.5% of A- horses that were also C+ (\(P = 0.0730\)). A small inverse trend (\(r = 0.286\)) was observed between the degree of ataxia and the concentrations of NO\(_x\) in the CSF of these horses (Fig. 3). The range of CSF NO\(_x\) concentrations for each ataxia score also appeared to narrow as the level of ataxia increased.

Sensitivity and specificity curves of cutoff values for NO\(_x\) concentrations in CSF were plotted to evaluate the ability of this assay to differentiate horses with EPM from horses without EPM (Fig. 4). The sensitivity and specificity curves intercepted at approximately 60% with a cutoff value of the CSF NO\(_x\) concentration of approximately 11 \(\mu M\) when the test for Abs in CSF was used and intercepted at 55% with a cutoff of 10 \(\mu M\) in the absence of a test for Abs in CSF. For the combination of the test for Abs in CSF and a cutoff value of 11 \(\mu M\) for the CSF NO\(_x\) concentration, the positive predictive value was 0.784 and the negative predictive value was 0.553. The specificities of the test for CSF NO\(_x\) concentrations in the presence and in the absence of a test for anti-\(S.\ neurona\) Abs in CSF were similar at the lower cutoff values, where the sensitivity was relatively low. However, diagnosis of EPM was more specific in conjunction with the detection of anti-\(S.\ neurona\) Abs in CSF at the intercept of the specificity and sensitivity curves.

![FIG. 1. CSF NO\(_x\) levels in horses experimentally infected with \(S.\ neurona\). Nine horses were divided into three groups that were subjected to transport stress, allowed to acclimate, or treated with dexamethasone (Dexameth.) prior to inoculation with sporocysts. CSF samples were collected before (open bars) and after (hatched bars) infection. Mean plus standard error CSF NO\(_x\) concentrations for each group, determined from assays of quadruplicate wells for each horse, are presented. Values with different superscripts are statistically different (\(P < 0.05\)).](image1)

![FIG. 2. CSF NO\(_x\) levels of horses naturally exposed to \(S.\ neurona\). CSF samples were assayed from 105 horses admitted to the OSU VTH from December 1997 through June 1998. Results are divided into four groups, based on ataxia scores of 0 (A-) or ≥1 (A+) and the presence (C+) or absence (C-) of anti-\(S.\ neurona\) Abs in the CSF. C+ A+ horses were considered to have EPM. The numbers of horses in the C+ A+, C- A+, C+ A-, and C- A- groups were 65, 16, 15, and 9, respectively. Least-squares means plus standard errors for each group, determined by assay of quadruplicate wells for each horse, are presented. The asterisk indicates a different value from that for the C+ A+ group, as determined by the mixed procedure described in the text (\(P < 0.05\)).](image2)

![FIG. 3. Inverse relationship between the levels of ataxia and CSF NO\(_x\) levels. The mean concentration of NO\(_x\) in the CSF of equine patients described in Fig. 2 were plotted according to their ataxia scores, and the linear regression was determined with Microsoft Excel software. The numbers of horses in each group with ataxia scores of 0 to 5 were 22, 9, 49, 18, 4, and 3, respectively.](image3)
DISCUSSION

A reduction in CSF NO\textsuperscript{\textsuperscript{\textsuperscript{\textsuperscript{-}}} levels after experimental exposure to \textit{S. neurona} was observed for transport-stressed horses, which had the highest ataxia scores, while increased CSF NO\textsuperscript{\textsuperscript{\textsuperscript{-}}} levels were observed for horses in the acclimated and dexamethasone-treated groups. In addition, CSF NO\textsuperscript{\textsuperscript{\textsuperscript{-}}} levels were lower in horses with naturally occurring cases of EPM (i.e., those horses that had \textit{S. neurona}-specific Abs in their CSF and that were ataxic) than in nonataxic horses that had \textit{S. neurona}-specific Abs in their CSF. Experimental exposure of horses to \textit{S. neurona} allowed the use of preinfection CSF from each horse as a negative control, the comparison of different preinfection treatments to invoke EPM, the use of a homogeneous source of hosts and parasite, and the observation of CNS lesions at necropsy. The study with experimentally infected horses was complemented by the natural exposure study that consisted of a much larger, heterogeneous, and outbred population of horses with clinical signs and asymptomatic, unexposed, or naturally exposed horses. The association of lower CSF NO\textsuperscript{\textsuperscript{-}} levels with clinical signs of EPM among horses with experimentally or naturally induced EPM indicated that this observation could be used to develop an experimental model for EPM that resembles the disease that occurs under natural conditions (30, 31).

Our results suggest that there is an increase in the level of NO production in the presence of \textit{S. neurona} infection when the hosts are not stressed under experimental conditions. These observations are supported by those of others who reported increased levels of iNOS mRNA and NO production in vitro and in vivo by mammalian cells, particularly macrophages, in the presence of IFN-\gamma and numerous pathogens including other parasitic protozoa such as the apicomplexans, \textit{Plasmodium falciparum}, \textit{Babesia bovis}, and \textit{Toxoplasma gondii}, as well as trypanosomes such as \textit{Leishmania major} and \textit{Trypanosoma cruzi} (14, 20, 23, 27, 33, 34). In the case of EPM, however, these results do not indicate that the production of increased levels of NO is a mechanism of pathogenesis. Indeed, a slight inverse relationship between the levels of ataxia and the levels of NO\textsuperscript{\textsuperscript{-}} in CSF appeared to occur. The results among the experimentally infected horses suggest that an increased level of NO production could have been associated with protection against \textit{S. neurona}. However, it is noteworthy that both the naturally exposed and unexposed horses with neurologic disease had lower CSF NO\textsuperscript{\textsuperscript{-}} levels than their neurologically normal counterparts. Thus, the association between a lack of clinical disease and higher levels of NO\textsuperscript{\textsuperscript{-}} in CSF may not be due to the enhanced parasiticidal activity of NO and its metabolites per se, but, rather, the decreased levels of NO production could be associated with the onset of neurologic disease.

Another explanation for these observations is that higher NO levels in horses with subclinical disease suppress an immunopathogenic response to \textit{S. neurona}, simultaneously reducing signs of ataxia and allowing the formation of parasitic lesions in the CNS. For example, NO production can affect the functional activity of interleukin-12 (IL-12), a cytokine that is required for priming of a type 1 helper T-cell response and IFN-\gamma production by NK and helper T cells (36). NO is required for transcription of the p40 subunit, but not the p35 subunit, of IL-12 in murine macrophages (28). However, high levels of NO production could result in the formation of p40 homodimers, which are antagonists of functionally active heterodimeric IL-12 (11). These observations are underscored by reports of increased steady-state levels of IL-12 p40 mRNA parallel to increased levels of iNOS mRNA in bovine macrophages stimulated in vitro with \textit{B. bovis} in the presence of IFN-\gamma (33) and of augmented IFN-\gamma production, an indication of active heterodimeric IL-12, by antigen-stimulated spleen- or lung-associated lymph node cells from iNOS knockout mice infected with the protozoal parasite \textit{Trypanosoma brucei} (15) or the trematode parasite \textit{Schistosoma mansoni} (17). In addition, increased iNOS activity has been associated with suppression of the wild-type murine cellular immune response to \textit{T. brucei}, as demonstrated by inhibition of spleen cell proliferation, which was partially reversed by an iNOS inhibitor (32). Thus, increased levels of NO production in response to \textit{S. neurona} might result in a less pathogenic host immune response in the CNS, albeit one that is less protective against infection with the parasite. Further studies are required to elucidate the precise role of NO in immune protection and neuropathogenesis associated with EPM.

The association between CSF NO\textsuperscript{\textsuperscript{-}} concentrations and ataxia are consistent with clinical observations by others who described ataxia and mild depression following exercise in experimental horses that were infused with the iNOS inhibitor 
\textit{N}^\text{\textsuperscript{\textsuperscript{-}}}-\textit{L}-nitro-arginine methyl ester (18, 24). Low levels of NO can lead to neurodegeneration through restricted cerebral blood flow (10, 35) and oxidative stress due to increased levels of superoxide production (38, 39). Reduction of the level of NO production can result from decreased levels of the NOS substrate \textit{L}-arginine or a NOS coenzyme, tetrahydrobiotin (BH4) (37). Reduced levels of NO metabolites have been
reported in association with several human neurologic diseases including cerebral malaria (2), Alzheimer’s disease, Parkinson’s disease, Huntington’s disease, and multiple-system atrophy (19). Decreased levels of BH4 have also been associated with Alzheimer’s and Parkinson’s diseases (3, 25). The cause of the decreased CSF NO\textsubscript{3} levels in horses with EPM appears to be either multifactorial (e.g., it can be caused by infection with S. neurona and stress) or a consequence of neurologic disease. These results suggest that therapeutic objectives directed at increasing or maintaining NO levels in the CNS, such as treatment with dexamethasone, L-arginine, and/or BH4, could be effective therapy for EPM.

This work demonstrates a measurable difference between stressed and nonstressed horses in the onset of clinical EPM and indicates the importance of stress in the development of this disease under natural conditions. Further work is needed to determine the role of the parasitic infection in the reduction of CSF NO\textsubscript{3} levels in horses with clinical EPM and to determine whether this observation is a cause or a consequence of the disease. Additionally, evaluation of the concentrations of NO\textsubscript{3} in the CSF of C+ A+ horses that respond to antiprotozoal treatment could facilitate determination of the utility of this assay as a test for the presence of disease and the response to treatment.

The combination of a test that determines the CSF NO\textsubscript{3} concentration and a test for Abs in CSF might increase the likelihood of a correct diagnosis and improve the prognosis with the advent of more effective treatments for EPM because an assay for determination of the concentration of CSF NO\textsubscript{3} could be used to objectively distinguish between horses with clinical EPM and those only exposed to the parasite. However, a test for anti-S. neurona Abs in CSF would still be necessary because C– A+ horses could not be distinguished from those with EPM by determination of the CSF NO\textsubscript{3} concentration alone. The presence of anti-S. neurona Abs and reduced levels of NO\textsubscript{3} in horse sera could also be evaluated as a potential diagnostic test for EPM. A better “gold standard” for the diagnosis of EPM would be valuable in further determining the value of a test that measures the concentration of NO\textsubscript{3} in CSF identifying horses with this disease and in predicting the efficacies of different treatments. The utility of a more specific and/or sensitive test for Abs in CSF and the detection of Abs and NO\textsubscript{3} in CSF among vaccinated horses that have not been exposed to S. neurona must also be determined.

ACKNOWLEDGMENTS

This work was supported by American Life Stock Insurance (Geneva, Ill.), the Department of Veterinary Preventive Medicine at The Ohio State University, The Ohio State University College of Veterinary Medicine Equine Research Funds, and the Ohio Quarter Horse Association.

We are grateful to W. C. Brown for helpful comments during the preparation of the manuscript and thank D. L. Grover and J. Stanek for technical assistance.

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