Lymphocyte Phenotype Subsets in the Cerebrospinal Fluid of Normal Horses and Horses with Equine Protozoal Myeloencephalitis*

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\section*{ABSTRACT}
The percentages of T-lymphocytes, lymphocyte subsets CD4+ and CD8+ T-cells, and lymphocyte adhesion molecule CD11a/CD18 were determined in the peripheral blood and cerebrospinal fluid (CSF) of seven normal horses and four horses with equine protozoal myeloencephalitis (EPM) using flow cytometry. There was a greater percentage of CD5+ cells in the CSF (79.0%) than in peripheral blood (67.0%), although this did not achieve statistical significance. Furthermore, the lymphocyte population in CSF comprises a significantly greater ($P = .01$) percentage of CD8+ T-cells, resulting in a decrease of the CD4/CD8 ratio. Lymphocyte phenotype subsets in peripheral blood or CSF from horses affected with EPM did not differ from normal horses, although CD5+ T-lymphocytes were seen in significantly greater numbers in the CSF of EPM-affected horses (93.2%) than in normal horses (79.0%).

\section*{INTRODUCTION}
Equine protozoal myeloencephalitis (EPM) is a serious condition of the central nervous system (CNS) of horses, leading to clinical signs of ataxia, muscular weakness, and stumbling.\textsuperscript{1} Currently, EPM is one of the most commonly diagnosed nervous system disorders of horses in the United States.\textsuperscript{1} A report from The Ohio State University indicates that 25% of all hors-
Many more horses may be exposed as demonstrated by a high seroprevalence (>50%). Equine protozoal myeloencephalitis is most commonly caused by the protozoan parasite *Sarcocystis neurona*, which invades the CNS, leading to necrosis and inflammation of nervous tissue. The nature of the clinical signs is a reflection of neuroanatomic localization of the parasite in that they are usually asymmetrical and multifocal.

Since the infection occurs within the CNS, the activity of the immune response, including lymphocyte phenotype subsets within this compartment, is important. Currently, the normal lymphocyte phenotype subset distribution within the cerebrospinal fluid (CSF) is not known in horses. The study reported here was performed to determine this distribution and to further determine if the phenotype distribution in normal horses differs in the peripheral blood or CSF of horses with EPM. The protocol described herein was reviewed and approved by the Virginia-Maryland Regional College of Veterinary Medicine Animal Care and Use Committee.

**MATERIALS AND METHODS**

**Animals**

Whole-blood and CSF samples were collected from 11 horses, including ten geldings and one mare, 3 to 12 years of age. Breeds represented were thoroughbred or thoroughbred cross (9), quarter horse (1), and paint (1). A standardized neurologic examination was performed to determine clinical neurologic status, and horses were scored using standardized criteria. Tests included standing cervical radiographs and routine CSF examination, including cell count, cell differential, and Western blot (WB) analysis for antibodies to *Sarcocystis neurona*.

Criteria for inclusion in the study as a normal horse required finding no neurologic deficits at the initial examination or during 6 months follow-up after completion of the study. In addition, analysis of CSF and cervical radiographs had to be within normal limits with no suggestion of any abnormality. Results of the WB analysis could be either negative or weakly positive, however, as many normal horses have been found to demonstrate a weakly positive response on this sensitive test. To ensure there was no damage to the blood-brain barrier or contamination of the CSF with peripheral blood, any sample with a red blood cell (RBC) count greater than 100 cells/µl was considered contaminated and not used for analysis.

Inclusion criteria for the EPM group required that the horses demonstrate neurologic deficits consistent with EPM (e.g., asymmetric ataxia, muscle wasting) of Grade 2 or greater (on a scale of 0 to 5) and no evidence of cord compression, canal narrowing, or other abnormalities by radiography of the cervical spine. In addition, if the evaluation of CSF found evidence of another disease, horses were not included. As in the normal horses, absence of peripheral blood contamination of the CSF was confirmed by RBC counts of less than 100 cells/µl.

A complete postmortem examination was performed on two neurologically normal and two EPM-affected horses at the conclusion of the study.

**Sample Collection**

Peripheral blood was collected from the jugular vein into evacuated tubes containing sodium EDTA as an anticoagulant. Cerebrospinal fluid was collected in a routine manner utilizing the atlanto-occipital space to minimize the possibility of peripheral blood contamination and to increase the yield of CSF collected. Briefly, horses were sedated with xylazine at 1.1 mg/kg body weight by intravenous injection. After adequate sedation was achieved, the horses were anesthetized with ke-
tamine by intravenous injection. The atlanto-occipital site was surgically prepared, and a minimum of 70 ml of CSF was collected with gentle aspiration via a 3.5-inch stiletted needle.

Sample Preparation
Whole-blood samples were maintained at room temperature until routine complete blood cell counts were performed using a commercial automated hematology analyzer. Cells were stained, and differential counts were performed manually on 100 cells by an experienced hematology technician. Cerebrospinal fluid cells (nucleated and nonnucleated) were enumerated using a counting chamber, and then a smear was prepared and stained following centrifugation. Differential counts were performed manually after counting at least ten cells. Each CSF sample was then divided into six or seven sterile 15-ml conical tubes, which were centrifuged at 200 × g for 25 minutes at 4°C. The supernatant was discarded, leaving approximately 1 ml of CSF, which was gently agitated, transferred to a sterile 2-ml polypropylene tube, and centrifuged at 200 × g for 5 minutes at 4°C. The supernatant was decanted, and the remaining sample was used for cell labeling.

Lymphocyte Labeling
Aliquots of whole blood and CSF were labeled with monoclonal antibodies as described below. Primary antibodies for CD4 T-cells (clone HB61A), CD8 T-cells (clone HT14A), and CD5 T-cells (clone HT23A) were obtained from VMRD, Inc. (Pullman, WA), and primary antibody for CD11a/CD18 (clone CVS 9) was obtained from Serotec (Oxford, England). All primary monoclonal antibodies were of the IgG1 isotype. The secondary antibody was fluorescein isothiocyanate (FITC)-conjugated goat antimouse IgG1. The panel of monoclonal antibodies used was limited in scope but was selected due to limitations in cell numbers available. Isotype-matched control negative antibody was used to label one aliquot of whole blood and CSF. For cell labeling, primary antibody was diluted to 40 µg/ml, with the exception of CD11a/CD18, which was used undiluted per manufacturer’s instructions. Secondary antibody was diluted 1:50. Whole blood (90 µl) was incubated at 4°C for 30 minutes with either 100 µl of diluted antibody or 20 µl of undiluted CD11a/CD18 monoclonal antibody. Cells were then washed twice with Hanks buffered salt solution (HBSS) containing 1% bovine serum albumin and 0.2% sodium azide. Secondary antibody (100 µl) was applied to all samples and incubated for 30 minutes at 4°C. Whole blood was then lysed by applying lysing solution. Whole-blood samples were washed twice and cells were resuspended in 500 µl of HBSS and analyzed by flow cytometry within 2 hours. Cerebrospinal fluid samples were labeled as above, but were washed between steps only once to avoid loss of cells.

Fluorescence-Activated Cell Sorter Analysis
All samples were analyzed using the FACSCalibur fluorescence-activated cell sorter (Becton Dickinson Immunocytometry Systems, San Jose, CA), and data acquisition and manipulation were performed using commercial desktop software (Cell Quest, Becton Dickinson). The fluorescence intensity for each monoclonal antibody was plotted as a histogram. For whole blood, 10,000 lymphocytes were analyzed after setting a gate utilizing light scatter properties (90° side scatter versus forward scatter). Cerebrospinal fluid cells were analyzed until the sample was exhausted or until 10,000 cells were analyzed, whichever occurred first.

Statistical Analysis
Results were evaluated using a computer-
based statistical analysis program (Minitab™, Minitab Inc., State College, PA). Descriptive statistics were performed as well as comparison of blood and CSF results using the two-tailed, paired Student’s t-test with significance preset at $P < .05$. Total cell numbers were also compared by a Student’s t-test. For comparisons in which data demonstrated unequal variance, the Satterthwaite approximation was performed. To compare peripheral blood and CSF lymphocyte subsets, only data from normal horses were analyzed to eliminate any confounding effects of disease upon the analysis.

## RESULTS

### Animal Findings

Of the 11 horses evaluated, seven were considered to be normal and four demonstrated compelling clinical evidence of EPM at the start of the study. Severity of ataxia was Grade 3 in three horses and Grade 2 in one horse. Gross postmortem and histopathologic evaluation of two of the abnormal horses provided additional evidence that EPM was the most likely diagnosis. Grossly, focal yellowish-tan areas were observed in the cervical spinal cord of both horses. Histopathologic evaluation revealed lymphocytic/plasmacytic perivascular cuffing and neuronal necrosis consistent with EPM in both animals. Gross and histologic evaluation of two selected normal horses failed to reveal any nervous system abnormalities. The other horses from the normal group remained clinically normal for 6 months following the end of the study.

### Hematology

Results of complete blood cell counts on peripheral blood and CSF are reported in Table 1. Briefly, no changes were noted between EPM-affected horses and normal horses with respect to total white blood cell (WBC) counts in the peripheral blood. Mean CSF cell counts were 0.28 cells/µl in normal horses and 1.25 cells/µl in horses with EPM. Cell counts in CSF ranged from 0 to 2 cells/µl in EPM-affected horses and 0 to 1 cell/µl in normal horses. Differential counts in CSF were 100%

<table>
<thead>
<tr>
<th>Cells Types</th>
<th>Normal Horses (n = 7)</th>
<th>EPM-affected Horses (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of Cells per µl</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>Range</td>
</tr>
<tr>
<td>Central nervous system (CNS)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total WBCs</td>
<td>9433</td>
<td>6400–11700</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>5050</td>
<td>3712–6634</td>
</tr>
<tr>
<td>Bands</td>
<td>36</td>
<td>0–214</td>
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<tr>
<td>Lymphocytes</td>
<td>3898</td>
<td>1792–6318</td>
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<tr>
<td>Monocytes</td>
<td>82</td>
<td>0–234</td>
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<tr>
<td>Eosinophils</td>
<td>247</td>
<td>82–856</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>CSF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total WBCs</td>
<td>0.28</td>
<td>0–1</td>
</tr>
</tbody>
</table>


TABLE 1. White Blood Cell (WBC) Counts in Peripheral Blood and Cerebrospinal Fluid (CSF) of Normal Horses and Horses with Equine Protozoal Myeloencephalitis (EPM)
mononuclear cells in all horses, except for one normal horse, in which the distribution was 80% mononuclear and 20% neutrophils. Red blood cell counts in CSF were below 10 cells/µl and routine clinical CSF analyses (total protein, glucose, creatine kinase concentration) were normal in all horses.

**Lymphocyte Phenotype Analysis**

Results of lymphocyte subset percentages in blood and CSF of normal horses are presented in Table 2. Notably, the CSF sample contained a uniform cell population based on light scatter properties. These cells were predominantly CD5+ T-cells (79.0%), which comprised only 67.0% of lymphocytes in the peripheral blood. CD8+ T-cells represented a significantly greater ($P = .001$) percentage of the total lymphocytes in CSF than in peripheral blood (23.4% versus 13.6%). CD4+ T-cells were also noted in greater percentage in CSF (59.6%) than in peripheral blood (51.4%); however, this did not achieve statistical significance ($P = .25$). The CD4/CD8 ratio was decreased in the CSF, primarily due to an increase in CD8+ T-cells ($P = .01$), with a minimal increase in CD4+ T-cells.

The total numbers of CSF cells were similar between the two groups (Table 3). Lymphocyte subset percentages did not differ significantly

### Table 2. Percentage of Labeled Cells in Peripheral Blood and Cerebrospinal Fluid (CSF) of Normal Horses and Horses With Equine Protozoal Myeloencephalitis (EPM)

<table>
<thead>
<tr>
<th>Cell Label</th>
<th>Normal Horses (n = 7)</th>
<th>EPM-affected Horses (n = 4)</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD5+</td>
<td>67.0 ± 9.6</td>
<td>79.0 ± 16.8</td>
<td>.14</td>
</tr>
<tr>
<td>CD4+</td>
<td>51.4 ± 7.9</td>
<td>59.6 ± 15.6</td>
<td>.25</td>
</tr>
<tr>
<td>CD8+</td>
<td>13.6 ± 3.7</td>
<td>23.4 ± 4.9</td>
<td>.001</td>
</tr>
<tr>
<td>CD11a/CD18</td>
<td>83.4 ± 13.1</td>
<td>98.2 ± 1.6</td>
<td>.06</td>
</tr>
<tr>
<td>CD4/CD8</td>
<td>3.9 ± 0.8</td>
<td>2.6 ± 0.8</td>
<td>.01</td>
</tr>
</tbody>
</table>

*Value for normal horses versus corresponding value for EPM-affected horses.
†Value for blood versus value for CSF in normal horses.

### Table 3. Total Cell Numbers Counted by Flea Cytometry in Cerebrospinal Fluid of Normal Horses and Horses With Equine Protozoal Myeloencephalitis (EPM)

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Normal Horses (n = 7)</th>
<th>EPM-affected Horses (n = 4)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD5+ T-cells</td>
<td>3838 ± 3426</td>
<td>3892 ± 2180</td>
<td>.98</td>
</tr>
<tr>
<td>CD4+ T-cells</td>
<td>5922 ± 3811</td>
<td>4246 ± 2834</td>
<td>.49</td>
</tr>
<tr>
<td>CD8+ T-cells</td>
<td>5085 ± 3064</td>
<td>4597 ± 2192</td>
<td>.80</td>
</tr>
<tr>
<td>CD11a/CD18</td>
<td>3543 ± 3420</td>
<td>3073 ± 1831</td>
<td>.84</td>
</tr>
</tbody>
</table>
between clinically normal horses and horses with EPM; however, there was a trend toward more CD8+ cells in the peripheral blood (18.2% versus 13.6%) and in CSF (26.9% versus 23.4%) of affected horses than in normal horses (Table 2). The CD4/CD8 ratio did not differ significantly in the peripheral blood or CSF between EPM-affected horses and normal horses, although the ratio in the peripheral blood of affected horses (2.9) was less than for normal animals (3.9). The percentage of lymphocytes that were CD11a/CD18+ was high in both CSF and peripheral blood. Although the percentage of CD11a/CD18+ cells in CSF was greater than the corresponding peripheral blood sample (98.2% versus 83.4%) in every horse examined, it did not achieve statistical significance ($P = .06$). The percentage of CD11a/CD18+ cells in peripheral blood or CSF did not differ between clinically normal or affected individuals. Representative flow cytometry results are presented in Figures 1A to 1C.

**DISCUSSION**

The study described here is the first evaluation of CSF lymphocyte subset analyses in horses. The very low cell counts typically observed in CSF (<5/µl) were overcome by the use of large volumes of CSF and the concentration method described. Although fewer cells were recovered than anticipated, numbers were adequate for completion of the study. The results from the peripheral blood analysis are similar to those previously described for horses, validating the techniques applied in this study.⁶,⁸ Lymphocyte subsets in CSF were markedly different from corresponding findings in peripheral blood. These findings are similar to those found in other species and humans, in which T-cells predominate in the CSF.⁹,¹⁰ The present findings differ, however, in that CD8+ T-cells are increased as a percentage of total CSF cell numbers, while in humans

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**Figure 1A**

**Figure 1B**

**Figure 1C**

**Figure 1.** Representative flow cytometry results from cerebrospinal fluid, showing typical forward scatter image (FSC-H) versus side scatter (SSC-H) (A). Side scatter image versus fluorescence (FL1-H) (B). Histogram showing the distinct positive-labeled population (C).
and other species, CD4+ T-cells tend to predominate in the CSF. CD4+ T-cells in the CSF of the horses in the present study did not differ markedly from those in peripheral blood, resulting in an overall decrease in the CSF CD4/CD8 ratio.

The finding of increased CD8+ cells in the CSF is similar to the observation that CD8+ T-cells are also increased in lung bronchoalveolar lavage fluid (BALF) when compared with numbers in peripheral blood. Decreased T-cells (as CD5+ cells), as well as a decreased CD4/CD8 ratio, were reported from other studies of equine lung BALF, similar to results obtained in the current study of CSF lymphocyte subsets.

Lymphocyte trafficking to the CNS and to other tissues is dependent on the expression of adhesion molecules on the surface of the lymphocyte and corresponding adhesion molecule ligands on endothelium. In vitro studies have suggested that LFA-1 with its ligand ICAM-1, as well as VLA-4 with its ligand VCAM, are key to migration of lymphocytes into the CNS. The equine homologue of LFA-1 is CD11a/CD18, which is expressed on a large percentage of peripheral blood lymphocytes and granulocytes. The widespread expression of CD11a/CD18 on cells within equine CSF suggests that cellular activation is needed prior to entry. Current data are not adequate to completely support this, however, and further studies are required to more fully investigate this observation.

The percentage of peripheral blood lymphocytes expressing CD11a/CD18 was not as high in the current study (83.4%) as it was in the original report by Zhang (>90%). The difference may be explained by methods employed in the preparation and analysis of the cells. Zhang analyzed peripheral blood mononuclear cells that had been prepared by ficoll-hypaque separation, while in the study reported here lysed whole blood was used. The whole-blood lysis method has been shown not to affect the results of other lymphocyte subtypes (CD5, CD4, and CD8), but has not been evaluated when labeling the CD11a/CD18 integrin.

White blood cell counts in peripheral blood were within normal limits and did not differ between normal horses and horses with EPM. White blood cell counts in CSF were within normal limits for both groups, although cell numbers were higher in horses with EPM. Although this difference did achieve statistical significance, the numbers of cells counted in samples from these horses were low enough that the difference is not likely to be of biological significance. Descriptions of CSF white blood cell findings in horses with EPM are not well represented in the literature, but an early report from Mayhew and DeLahunta described increased WBCs in the CSF of horses with EPM. This increase was seen only in samples collected from the lumbosacral site, however, and was associated with a high number of RBCs in the sample (1890/µl). Hence, peripheral blood contamination may have artificially increased the WBC count in that report. Other reports indicate that increased WBC counts in CSF are rarely seen, but are usually a mononuclear pleocytosis when observed, with eosinophils or neutrophils rarely present.

The distribution of lymphocyte subsets in the CSF of horses with EPM has not been examined previously. In the study presented here, CSF CD5+ T-cell percentages were numerically greater in horses with EPM than in normal horses, but the difference was not significant. Further studies with a larger sample size are warranted to fully evaluate this observation. In a previous study of peripheral blood lymphocyte subsets in EPM-positive horses, symptomatic horses had a lower peripheral blood CD4+ percentage than asymptomatic animals, with no difference in CD8+ cell percentages.
No difference was noted in the CD4+ cell percentages of the horses in the present study; however, the test sample was small and case selection differed, making direct comparison difficult. The present study reports the normal distribution of lymphocyte phenotype subsets in the CSF of horses and describes the findings of lymphocyte phenotype analysis of peripheral blood and CSF in horses with EPM. Significant differences were noted between the distribution of cells in the peripheral blood and CSF of normal horses. In addition, no changes were noted when comparing the findings of normal horses with those of EPM-affected horses. The differences in the T-cell repertoire of equine CSF may have a significant impact upon the ability of the horse to clear infection, and further studies are necessary to establish the nature of the equine lymphocyte response to \textit{S. neurona} infection.

\textbf{REFERENCES}

2. Reed S, Granstrom D, Rivas L: Results of cerebrospinal fluid analysis in 119 horses testing positive to the Western blot test on both serum and CSF to equine protozoal myeloencephalitis. \textit{Proc AAEP} 40:199, 1994.