

Equine Protozoal Myeloencephalitis.  
Preliminary Investigation of Protozoan-Host Interaction in the Horse.

by

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(ABSTRACT)

Equine Protozoal Myeloencephalitis (EPM) is the most frequently diagnosed neurologic disorder of horses in the United States, and it is caused by the protozoan organism *Sarcocystis neurona*. The disease has a profound impact on the American Horse Industry. This impact includes prolonged and expensive treatment without a guaranteed return to a previous level of exercise for the individual horse. Poor response to, and the prolonged duration of treatment, may suggest an immune mediated impairment of host response. There is limited information about the direct interaction between the pathogen and the host.

In two *in vitro* experiments we investigated a) whether the presence of the protozoan organism can influence mitogen-stimulated peripheral blood mononuclear cells (PBMCs), suggesting a direct influence of the protozoan organism on cells of the immune system, and b) if cerebrospinal fluid (CSF) from horses with EPM has an effect on mitogen stimulated PBMCs, suggesting that the Central Nervous System microenvironment may influence the course of the disease.

**Experiment 1**

Mitogen-stimulated PBMCs from EPM affected and control horses were co-cultured with fragments of freeze-thawed bovine turbinate cells, that were infected with *S. neurona* merozoites. Compared to controls PBMCs co-cultured with *S. neurona* fragments were the only cells that showed a decreased proliferative ( $p < 0.05$ ). A difference between EPM affected and control horses could not be detected ( $p > 0.05$ ). These results may

implicate that the persistence of *S. neurona* infection in the horse's CNS is, in part, due to a pathogen-derived mechanism that attenuates the host's immune response.

## **Experiment 2**

Mitogen-stimulated PBMCs from a horse affected with EPM and a control were co-cultured in the presence of cerebrospinal fluid (CSF) from EPM affected horses and uninfected controls. Prior to co-culture the CSF was fractionated by a filtration process over two microfilter units. An identical volume of NaCl (0.9%) served as control for the volume of CSF that was added. The proliferation assay revealed a deviation of the response depending on cell donor and CSF fraction that was used. The effect was independent from the protein concentration of the CSF fractions, and a decrease in lymphocyte proliferation was not caused by increased cellular death. This suggests the presence of substances within the CSF which have a stimulatory or suppressive influence on the cells in culture. The effect was cell donor dependent which implies a difference in lymphocyte subsets between the two horses that were used.

## Dedication

Wie alles sich zum Ganzen webt,  
Eins in dem andern wirkt und lebt!  
Wie Himmelskräfte auf und nieder steigen  
Und sich die goldnen Eimer reichen!  
Mit segenduftenden Schwingen  
Vom Himmel durch die Erde dringen,  
Harmonisch all das All durchklingen!

Welch Schauspiel! Aber ach! ein Schauspiel nur!  
Wo faß ich dich, unendliche Natur?

Johann Wolfgang von Goethe, Faust, 1. Akt

Dedicated to Harriet and William. For their friendship, laughter and Southern Hospitality.

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## List of Abbreviations:

APC	antigen presenting cell
BBB	blood-brain barrier
CNS	central nervous system
ConA	concanavalin A
CPM	counts per minute
CSF	cerebro-spinal fluid
EPM	Equine Protozoal Myeloencephalitis
ICAM	intercellular adhesion molecule
IFN-	Interferon gamma
Ig	immunoglobulin
IFA	immune fluorescence assay
IL	interleukin
IPS	immune privileged site
MHC	major histocompatibility complex
-MSH	melanocyte stimulating hormone
PBL	peripheral blood lymphocyte
PCR	polymerase chain reaction
POMC	proopiomelanocortin
RES	reticulo-endothelial system
RNA	ribonucleic acid
<i>S. neurona</i>	<i>Sarcocystis neurona</i>
TGF-	transforming growth factor beta
TH T	helper cell
TNF-	tumor necrosis factor
VCAM	vascular cell adhesion molecule
VIP	vasoactive intestinal peptide
VLA	very late activation molecules
WB	Western Blot

## 1. Introduction

Equine Protozoal Myeloencephalitis (EPM) is currently the most frequently diagnosed neurologic disorder of horses in the United States. The disease, which is caused by the protozoan organism *Sarcocystis neurona* has a profound impact on the American Horse Industry. The monthly costs for a recommended treatment for an adult horse with a body weight of about 500 kg currently ranges at about \$150 - 200 a month. Recommended treatment time can be six months or longer. It is not guaranteed that the horse will recover to its previous level of exercise, and horses on recommended treatment still can become recumbent, followed by death or chronic debilitation.

*Sarcocystis neurona* has a complicated life-cycle. Usually asexual meront stages are found in the musculature of birds (intermediate host), while the sexual replication takes place in the intestinal tract of the American opossum (*Didelphis virginiana*), which is the definitive host. The horse is an aberrant host. The protozoan penetrates the central nervous system (CNS) and replicates for an unknown number of cycles. The lesions caused by inflammation and tissue destruction are responsible for the clinical signs of EPM. An immunoblot assay (Western blot), which is a cornerstone in the diagnosis of EPM, detects antibodies against *S. neurona* merozoites in cerebro-spinal fluid (CSF). The currently recommended treatment is a combination of sulfadiazine, a competitive inhibitor of the prokaryotic dihydropteroate synthase, and pyrimethamine which is a dihydrofolate reductase inhibitor. Horses do show clinical improvement; however, despite long-term treatment (months to years), the immunoblot result on CSF may persistently demonstrate antibodies against the protozoan organism. Explanations for the persistent presence of antibodies in the CNS include the development of drug resistance of the protozoan, reinfection, persistent infection, and/or an impairment of the immune system to eliminate the protozoan organism from the CNS. It is not known whether the protozoan actively targets the CNS, or whether CNS infection is random. The CNS, however, is an immune

privileged site (IPS), where the extent of inflammation is ameliorated in order to protect tissue function. This phenomenon forms an obstacle in host defense, which might maintain the presence of viable merozoites stages within the CNS.

The objective of this study was to determine if protozoan fragments (Experiment 1) or cerebrospinal fluid (CSF) from affected horses (Experiment 2) have an effect on an *in vitro* concanavalin A (Con A) dependent lymphoblastogenesis assay.

## **2. Literature Review - Equine Protozoal Myeloencephalitis**

### **A. Introduction and History**

Equine Protozoal Myeloencephalitis (EPM) is currently the most frequently diagnosed disease of the central nervous system (CNS) of horses in the United States. EPM can cause a focal, multifocal or diffuse myeloencephalitis. The most common presenting clinical sign is an asymmetric ataxia; however, due to multifocal infection, the clinical signs can vary, affecting each level of the CNS and causing both spinal and/or brain signs (Fenger 1997b). Within the US there are regional differences in seroprevalence. In endemic areas the seroprevalence ranges between 40 and 50% (MacKay 1997); however, seroprevalence does not correlate with clinical signs. To date it is unknown how many seropositive horses exhibit clinical signs of EPM.

The syndrome of focal myelitis- encephalitis was first described by Rooney et al. (1969). In 1974 the disease 'Equine Encephalomyelitis' was attributed to a protozoan organism found within the lesions, which was initially identified and described as a "*Toxoplasma* -like organism" (Beech 1974, Dubey 1974). Later it became unlikely that the lesions were caused by a *Toxoplasma spp.*. Based upon morphologic studies of the asexual stages of the protozoan organism within lesions of post-mortem specimen it resembled the asexual replication of a *Sarcocystis* species rather than a *Toxoplasma spp.* This was based upon the observation of replication within the host cell (endopolygeny), and the absence of a parasitophorous vacuole and rhoptries (Simpson 1980, Dubey 1991).

The etiologic agent of Equine Protozoal Myeloencephalitis was named *Sarcocystis neurona* based on morphologic studies, cross reactivity with *Sarcocystis cruzi* antiserum, and absence of reactivity to sera against *Toxoplasma gondii*, *Hammondia*

Phylum:	APICOMPLEXA
Class:	Sporozoa
Subclass:	Coccidiasina
Order:	Eucoccidiorida
Suborder:	Eimeriorina
Family:	Sarcocystidae
Subfamily:	Sarcocystinae
Genus:	<i>Sarcocystis</i>

Fig 1: Phylogenetic Classification of *Sarcocystis spp.* (Dubey 1989)

*hammondi*, *Caryospora bigenetica*, and *Neospora caninum* (Dubey 1991). Davis and coworkers (1991) cultured *S. neurona* from the spinal cord of a horse with EPM, and followed the asexual part of the life cycle in vitro. The phylogenetic relationship (Fig. 1) of *S. neurona* to other members of the family *Sarcocystidae* has been elucidated based on small subunit ribosomal RNA gene sequencing (Fenger 1994), and has been reviewed in detail by Tenter (1995).

## B. Epidemiology

EPM has been described in North, Central and South America. Sporadic reports from Europe identified EPM in horses that were exported from the US. Apparently the disease cannot spread in absence of its definitive host. Seroprevalence of antibodies to *S. neurona* was evaluated with the introduction of the immunoblot (Western Blot) assay for antibody analysis. Seroprevalence in horses in central Kentucky and Ohio was found at about 20% (Rush-Moore 1995), while a recent review article indicated a seroprevalence of 40% to 50% within the eastern United States (MacKay 1997). These findings were based upon three different publications where horses in different geographic regions were evaluated (Oregon, Ohio, Pennsylvania). All studies confirmed a higher seroprevalence in older horses, implicating an increased likelihood of exposure with increasing age. The studies in Oregon and Pennsylvania included only horses that did not show clinical signs of Equine Protozoal Myeloencephalitis. In the seroprevalence study in Ohio samples

submitted for Equine Infectious Anemia-testing were separately analyzed for *S. neurona* antibodies. The sera were randomly selected and a history of clinical EPM was not known. Besides an overall seroprevalence of 45% in Oregon, the investigators also found regional differences of seroprevalence within the State. The seroprevalence was lower in the more arid eastern parts of the state (22%) vs. 65% in the coastal area. Gender, breed, and housing type were not associated with differences in seroprevalence, however seroprevalence consistently increased with age (Blythe 1997). Similar results were obtained in Pennsylvania. The overall prevalence was 45.3%, and a relationship with gender was not found. Again, seroprevalence was higher in older horses (Bentz 1997). The study performed in Ohio found an overall seroprevalence of 53%. Regional differences in seroprevalence correlated to the number of days below freezing with a lower seroprevalence in areas with more days a year below freezing. A linear relationship was found between age and seroprevalence of antibodies against *S. neurona*. The youngest seropositive horse in this study was 5 months old (Saville 1997). The seroprevalence in wild mustangs in Utah is less than 10% (Fenger 1997), which is presumed to be related to the relative paucity of the suspected definitive host in this area. A breed predilection, however, or environmental influences on infectivity of sporocysts could not be ruled out.

It is unknown how many seropositive horses exhibit clinical signs of the infection. It has been reported that the clinical disease seems overrepresented in Standardbreds, Thoroughbreds, and Quarter Horses (Fayer 1990), which is in contrast to the findings of several epidemiologic studies reported above. With the exception of one report of EPM in a pony (Dubey 1986), it has been proposed that ponies might be less susceptible to develop EPM (Granstrom 1992). Gardiner-Boy and coworkers (1990) reported that 87.5% of cases with EPM occurred in horses between 1 and 6 years of age. These results were based on histopathologically confirmed cases, which do not necessarily reflect seroprevalence. In a study of 364 histologically confirmed cases, 61.8% were younger than 4 years of age (Fayer 1990). Both reports suggest that young horses are more likely to develop clinical

signs of EPM. The youngest horse with clinical signs of EPM was 2 months old. Infectivity experiments showed an incubation period of 24 to 42 days for the development of clinical disease in combination with detection of intrathecally produced antibodies in cerebrospinal fluid (Fenger 1997). However, outbreaks of EPM in exported Thoroughbreds have occurred 1 year after leaving the United States. Seemingly protozoan stages can remain within the body for several months before they cause actual disease.

In summary, seroprevalence is high, and it is suggested to concur with the presence of the definitive host of *S. neurona*. Environmental conditions (temperature, humidity) can influence the survival of infectious stages in the environment. Seroprevalence is higher in older horses, based on increased likelihood of exposure to infectious environmental stages of the disease. The disease as an entity (presence of clinical signs) has been reported more frequently in younger horses, which could be due to disease recognition in relation to their economic potential. A breed predilection seems likely based on published reports. Standardbreds, Thoroughbreds, Quarter Horses seemed more likely to develop clinical disease than ponies (Granstrom 1994, Reed 1996, Saville 1997).

#### C. Life cycle of *Sarcocystis neurona*:

Members of the family *Sarcocystidae* classically have a two host, heteroxenous life cycle. The two hosts typically have a predator-prey relationship (Fig. 2). The sexual stages, characterized by the formation of micro and macrogamont stages within the intestinal tract (gamogony and sporogony) of the definitive host, results in the production and release of infectious sporocysts with the host's feces. Sporocysts are ingested by the intermediate host, where the asexual replication (endopolygeny) takes place within endothelial cells, followed by the formation of tissue cysts (endodyogeny), normally in muscle tissue. The numbers of schizont generations before the tissue cysts are formed are different and distinct between *Sarcocystis* species. The tissue cysts are infectious for the definitive host (Dubey 1989, Tenter 1995).

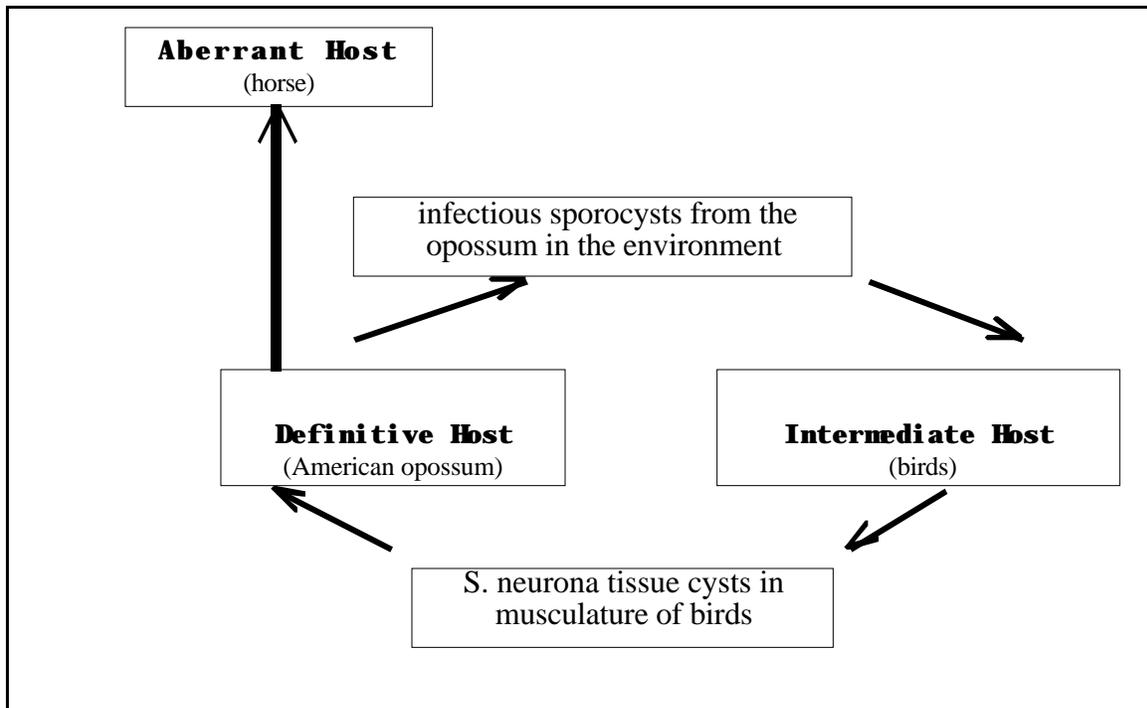


Fig. 2: Life cycle of *Sarcocystis neurona*

The complete life cycle of *Sarcocystis neurona* was not elucidated until 1997. Cross-infection experiments between the opossum, birds and the horse, identified the opossum as the definitive host. Birds were found to be the intermediate hosts, and the horse is an aberrant host. Infections from horse to horse are not possible, and opossums cannot be infected from horses because the meront-schizont stages do not mature. Infectious cyst stages have not been found in the CNS of horses. The disease is limited to the Americas, presumably because the opossum (*Didelphis virginiana*) is native to the Americas only. Fenger et al. (1995, 1997a) and Dame et al. (1995) identified the opossum as the putative definitive host of *Sarcocystis neurona*. Small subunit RNA (SSURNA) nucleotide sequence of *Sarcocystis neurona*, retrieved from spinal cord cultures, yielded a 99.89% identity with sporocysts derived from an opossum. However, the gene sequence identity (SSURNA) was also greater than 99% when *N. caninum* and *T. gondii* were compared, and therefore even close relationships among SSURNA gene sequence cannot

be considered conclusive evidence that two species are actually the same (Fenger 1997a). In an infectious model *Sarcocystis spp.* sporocysts from opossums were fed to weanling horses which exhibited signs of Equine Protozoal Myeloencephalitis and seroconversion after a post inoculation period of 28 to 42 days (Fenger 1997a).

In summary, the opossum is the definitive host for *S. neurona*. Sporocysts which were isolated from the intestine of opossums were infectious for horses and for birds. It is possible however, that different *Sarcocystis* subspecies use the opossum as their definitive host. Important for control of the disease is that horses are aberrant hosts for the protozoan. Tissue cysts, infectious for the definitive host are so far not identified in the horse.

#### D. Clinical Findings in EPM

The most common presenting clinical sign is asymmetric ataxia. Due to the presence of lesions, the presentation can vary, depending upon the location of the organism within the CNS. Rooney et al. (1969) described lesions predominantly in the lumbo-sacral spine. While Rooney was unaware of the protozoan organism that caused the lesions, he described the asymmetry and the focal character of the lesions. Mayhew (1978) found the majority of lesions in the cervical spinal cord. However, he included only those cases where protozoan stages were identified using light microscopy. Other clinical signs comprise lower motor unit deficits in all limbs leading to focal muscle atrophy. Each level of the CNS can become infected, however, and cranial nerve deficits, seizures and/or abnormal behavior can be associated with EPM (Fenger 1997b). Disease of the autonomic nervous system has not been reported.

*S. neurona* appears to be able to invade the central nervous system of a horse, by an unknown mechanism. Currently it is suggested that one cycle of asexual reproduction occurs in the endothelium of a peripheral vessel, including the endothelium of the cerebral vasculature (Liang 1998). The merozoite might also be transported into the CNS by an intracellular transport mechanism (Trojan Horse hypothesis), as described for *Toxoplasma*

*gondii*, or *Listeria monocytogenes*. Mononucleated cells serve hereby as a transport vehicle which carries infectious organisms into the CNS during the cells' routine surveillance of the CNS (Keane 1997). Lesions within the CNS are caused by the invading and subsequently replicating protozoan organism. Normally the number of schizont generations within an intermediate host are specific depending on the *Sarcocystis* species. *S. falcatula*, a closely related *Sarcocystis* spp. which also uses the opossum as a definitive host, seems to have an undefined, large number of schizont-to schizont cycles before tissue cysts form in its intermediate host (Dubey 1989). Based on a close phylogenetic relationship between *S. neurona* and *S. falcatula*, this might also be true for *S. neurona*.

Liang et al. (1998) demonstrated specific serum antibodies of horses against two membrane associated structures of the *S. neurona* merozoite, which could prevent new cellular infection or penetration of the blood brain barrier. However, the extracellular phase of merozoites in EPM is considered short, and it is doubtful whether this mechanism is effective in limiting further infection.

#### E. Ante Mortem Diagnosis

A diagnosis of EPM is currently based upon the presence of clinical (neurologic) signs, a positive Western Blot (WB) assay on a cerebrospinal fluid sample (CSF), and an increase of the CSF index (see under) without an increase of the permeability of the blood-brain barrier (BBB). Detection of antibody in the serum is an indicator of exposure only. PCR testing for small subunit DNA of the protozoan is available and helps to substantiate the diagnosis (Granstrom 1994b, Marsh 1996). The PCR on CSF, however, is rarely positive in clinical cases. An immunofluorescent antibody assay (IFA) uses *S. cruzi* as the antigen and relies on detection of cross reactivity of antibody between *S. cruzi* and *S. neurona* (MacFadden 1992). Agreement between the immunoblot and IFA tests is best at

higher concentrations of antibodies (Fenger 1997d). Published results on test sensitivity, specificity and predictive value of either PCR or IFA are not available.

#### Immunoblot Assay (Western Blot):

In general a Western blot (WB) is a procedure to identify a protein antigen that is in a mixture with other antigens (Abbas 1994). The antigen for the Western blot which is used for EPM testing originates from *S. neurona* merozoites that were cultured from the spinal cord of a horse with EPM (Davis 1991, Granstrom 1992). Antigen (lysed *Sarcocystis neurona* merozoite particles) is first separated, typically by sodiumdodecyl sulfate gel electrophoresis. The proteins are then transferred onto a support membrane, where the position of the antigen can be detected by binding antibody (Abbas 1994). With current EPM WB testing specific antibodies in serum or CSF are identified when binding to the antigen on the support membrane. Objectives during standardization of the test were to find specific antigens unique to *S. neurona*, which can be used for diagnosis of the disease by recognition of a distinct pattern of antigen determinants. Eight proteins were detected by *S. neurona* antiserum and/or serum from EPM-infected horses (Granstrom 1993). Currently at least three different laboratories provide an immunoblot assay for EPM testing. Their differences in methodology are based on recognition of different band or blot patterns, and/or are based on different antigen sources (Liang 1998, Fenger personal comm.). Reported sensitivity and specificity for the Western Blot assay from one of the laboratories is approximately 90% (Reed 1996). A WB test is a qualitative test which indicates the presence of specific antibodies in a variety of body fluids or suspensions. A WB does not quantitate the amount of antibody that is produced.

#### Polymerase Chain Reaction (PCR):

PCR testing is a method to amplify DNA or RNA strains in samples. The amplification increases the likelihood of detecting specific DNA or RNA. Consequentially a

PCR is a highly sensitive but qualitative test, and cannot be used to quantitate the infectious challenge. Two different PCR tests are available (Granstrom 1994b, Marsh 1996).

Granstrom's 'Kentucky' isolate of *S. neurona* differs in only 2 base pairs from a 'California' *S. neurona* isolate. A positive test result confirms the presence of protozoan DNA in the sample. As the protozoan organism is normally found intracellularly the likelihood of a positive PCR result is small. The PCR for *S. neurona* has a very high sensitivity; however, specificity is low due to a poor recovery of DNA within a CSF sample. Precise sensitivity and specificity of this test have not been published.

#### Cerebrospinal Fluid Indices: Albumin Quotient and IgG-Index

The Western blot assay on CSF can be falsely positive, meaning the test result is positive, however, the tested animal does not truly have EPM. False positive test results can be caused by blood contamination during the sampling procedure of a seropositive horse, or in case of a 'breached' blood-brain barrier (BBB), where serum antibodies can diffuse through a more permeable BBB. An increased permeability is seen during other infectious or pathologic conditions of the CNS (Equine Herpes Myelitis, bacterial meningitis, neoplasia). Either one can result in a positive immunoblot result on a CSF sample due to influx of *S. neurona* specific antibody from the peripheral blood into the CSF. The Albumin Quotient and the IgG-index help to decrease the number of false positive test results. To calculate these indices, it is necessary to determine the total IgG concentration in serum and CSF, and the albumin concentration in serum and CSF. The albumin quotient is then calculated by dividing the CSF albumin concentration by serum albumin concentration. An IgG-index is calculated by dividing the amount of IgG measured in CSF by IgG concentration in peripheral blood, multiplied by the albumin quotient (Fig. 3).

CSF indices can be used to determine if antibodies that were detected originate from the peripheral blood, or whether they are produced intrathecally. The calculation of an IgG index has been proposed as a quantitative assessment of an immune response within the CNS (Andrews 1990). An increased IgG-index is an indicator of intrathecal antibody production. It is the only semi-quantitative assessment of intrathecal antibody production available, and might be useful as an indicator of progress made during treatment for EPM (Andrews 1997). Cohen et al. confirmed the usefulness of the albumin quotient in interpretation of a positive immunoblot result for *S. neurona* on CSF; however, the usefulness of the IgG-index in clinical situations was questioned (Cohen 1997a, b), because the increase in IgG concentration may be due to other diseases that may clinically resemble EPM.

<b>Albumin Quotient (AQ):</b>	
$\frac{\text{Albumin CSF}}{\text{Albumin Serum}} \times 100$	normal: 0 - 2.2
<b>IgG-Index:</b>	
$\frac{\text{IgG (CSF)}}{\text{IgG (serum)}} \times \text{AQ}$	normal: 0.1 - 0.3
Fig. 3: CSF-indices (Andrews 1990) and normal values for adult horses (EBI 1997)	

F. Post-mortem diagnosis

Macroscopically focal hemorrhage and malacia can be detected visually upon sectioning of the samples. Histopathologic examination may show merozoites and/or schizonts in neuropile, microglia, macrophages and vascular endothelial cells of the CNS using routine staining techniques (hematoxylin/eosin). The protozoan stages, however, are difficult to identify by routine staining techniques only. Immunohistochemistry using an avidin-biotin complex immunoperoxidase method to demonstrate *S. neurona*, provides a higher yield, finding different parasitic stages within the CNS (Hamir 1993). Whether the PCR can be used detecting stages of the protozoan within homogenized tissue samples has not been investigated. Asymmetric non-suppurative inflammation with lymphoid perivascular cuffing, gliosis, and fiber degeneration in ascending and descending white

matter pathways cranial and caudal to the lesions are common findings. These findings are not pathognomonic for EPM however, unless protozoan stages are found within the lesions (Mayhew 1978).

In summary, the diagnosis of EPM remains challenging. Currently a test in which results correspond with the number of infectious organisms and the extent of damage to the CNS caused by these organisms is not available. The WB is a very sensitive test which detects small amounts of specific antibodies against *S. neurona.*, however, it is currently not explained why a majority of horses with stable clinical signs, and after a treatment period of a minimum of 6 months, still harbor specific antibodies within their CSF. It is essential to distinguish between peripheral blood antibodies and intrathecally produced antibodies. The situation in which a positive WB test results after a CSF collection with a normal albumin quotient and IgG index demonstrates the presence of specific (anti-*S. neurona*) antibodies in the sample will be used as an example for a short further discussion: The origin of these antibodies could be from minor blood contamination which was not noticed during the sampling procedure, from true intrathecal antibody production, or from passive diffusion of antibodies from the peripheral blood into the CNS.

Minor blood contamination of the sample could be due to damage of small blood vessels, followed by contamination of the spinal needle with blood, which is used for the CSF collection. Currently it is being investigated how much peripheral blood is necessary to cause a positive WB test result on a CSF sample (Reed, personal communication).

Intrathecal production of specific antibodies is thought to be due to the presence of the protozoan organism; however, it is postulated also that non-viable remnants of the protozoan remaining within the CNS after a course of treatment (see under) remain immunogenic. Intrathecal production of antibodies is the result of a migration of antigen specific B-Lymphocytes into the CNS. These cells initiate the production of antibodies when they encounter their specific antigen (Hickey 1997). Currently we do not know how

long the antibody producing cells remain within the CNS, nor how much antigenic mass is required to stimulate antibody production.

The possibility of passive antibody diffusion over the BBB is not only discussed among those interested in *S. neurona*, but is of importance in central nervous inflammatory disease. Streilein et al. (1997) believes that the immunoglobulins found in aqueous humor or CSF in the absence of disease represent the simple diffusion of these antibodies across the local BBB. He mentions that breaches in the BBB exist normally at the circumventricular organs of the brain and at the root of the iris in the eye. Cserr and Knopf (1997), however, believe that the fenestration of the cerebral endothelial cells at the circumventricular organs do not form a gateway for the entrance of antibody from the peripheral blood. They do not believe that the fenestration between the endothelial cells are large enough for immunoglobulins to pass through, and that passive diffusion of antibodies actually does not occur. They base their theory of strict intrathecal production of immunoglobulins on concentration differences in antibody isotypes within the CNS, and the inability to detect radioactive labeled protein molecules and immunoglobulins within the CNS after injection into the peripheral blood stream.

The WB seemingly remains positive in a vast majority of cases after treatment. In those cases of a normal AQ and IgG-index it is unknown whether the protozoan is still within the CNS, or whether these findings indicate the passive diffusion of antibody into the CNS. The IgG index is not sensitive enough, because it measures the total amount of antibody. Fenger (1998) proposed a dilution model where repeat WB evaluations after diluting CSF and serum indicate a disproportion of antibody concentrations between CSF and serum that cannot be explained by diffusion of antibodies over the BBB. In those cases where a disproportion or a larger than expected amount of CSF antibodies are found, there is true (intrathecal) production of antibodies, hence, ongoing and active infection. Results, however, are not yet available.

## G. Treatment

EPM has been treated successfully with a sulfonamide antibiotic and a 2,4-diaminopyrimidine, in particular pyrimethamine, a preparation previously used for the treatment of malaria in humans. The sulfonamides and the 2,4-diaminopyrimidines (pyrimethamine and trimethoprim) interfere with the production of tetrahydrofolate which is necessary for microbial RNA production (see figure 4). The sulfonamides competitively inhibit the microbial enzyme dihydropteroate synthase, which catalyzes the formation of dihydropteroate from para-aminobenzoic acid (PABA). The 2,4-diaminopyrimidines inhibit dihydrofolate reductase, an enzyme necessary for the synthesis of tetrahydrofolate from dihydrofolate. Tetrahydrofolate is an essential coenzyme necessary in the production of thymidine, purines and certain amino acids.

The selective bacteriostatic action of the sulfonamides and the diaminopyrimidines depends upon differences between the mammalian and bacterial cell in the source of folic acid. Susceptible microorganisms must synthesize folic acid, whereas mammalian cells use preformed folic acid. The conversion of dihydrofolate to tetrahydrofolate in the mammalian cell involves the same enzyme (dihydrofolate reductase). Its affinity for the 2,4-diaminopyrimidines is lower for the mammalian cell than the microbial cell; however, differences regarding the affinity of the 2,4-diaminopyrimidines for the mammalian dihydrofolate reductase exist. Pyrimethamine is more active against the mammalian enzyme than trimethoprim, and therefore can cause folic acid deficiencies. Supplementation of folic acid or folic acid might be necessary if clinical signs of folic acid deficiencies develop (Adams 1995). Folate supplementation counteracts the effective absorption of the 2,4-diaminopyrimidines (Zimmerman 1987), and supplementation of folic/folinic acid and treatment with 2,4-diaminopyrimidines should be separated.

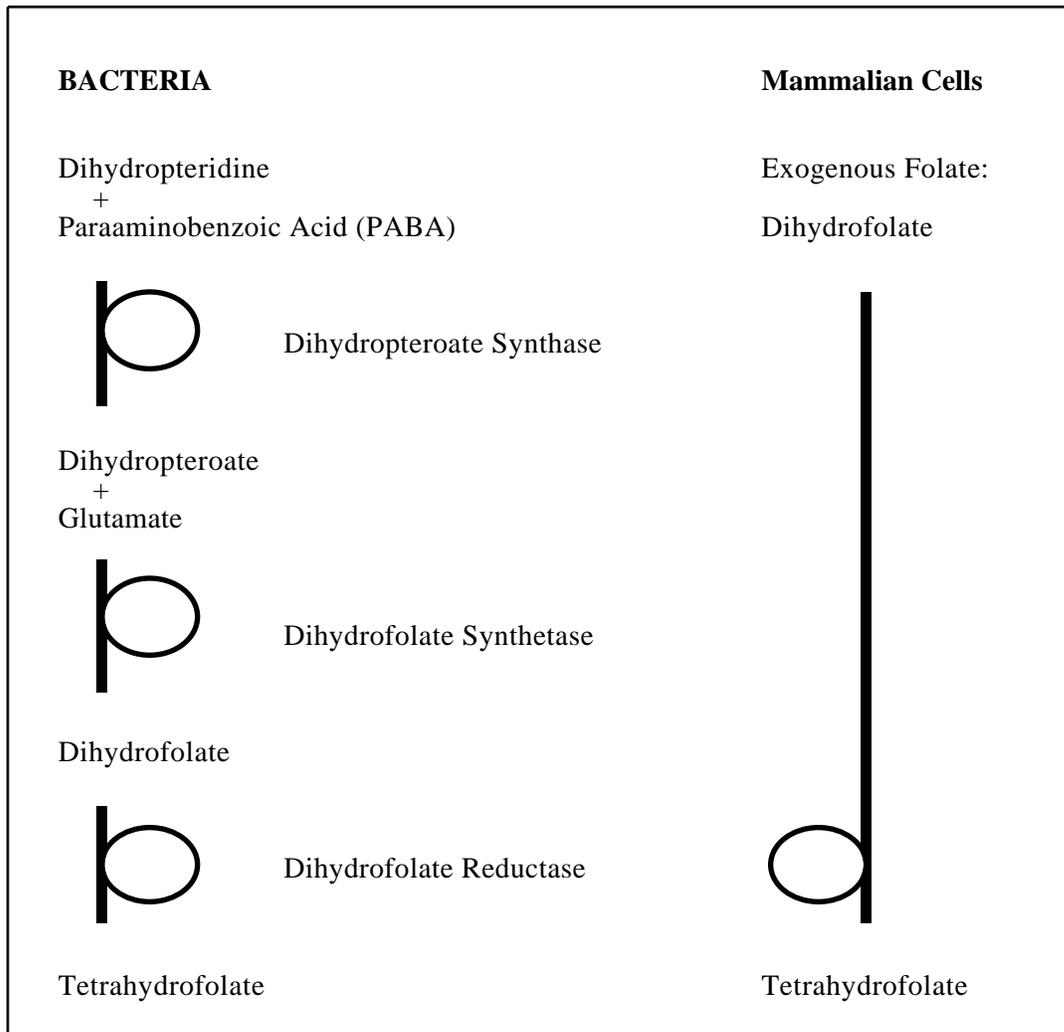


Fig. 4: Tetrahydrofolate production in Bacteria and Mammalian Species

Historically horses were treated with a combination of sulfamethoxazole-trimethoprim (30 mg/kg bw. twice daily) and pyrimethamine (0.5 - 1.5 mg/kg bw once daily). A combination of sulfadiazine (20 mg/kg bw. once daily) and pyrimethamine (0.5 - 1.5 mg/kg bw. once daily) has been proposed recently (Fenger 1997d,e), a dosage which was extrapolated from treatment for human toxoplasmosis. Pharmacokinetics and penetration of pyrimethamine into the cerebrospinal fluid after multiple doses has been evaluated, however, not in combination with a sulfonamide antibiotic. A dose of 1 mg/kg

once daily seemed necessary to achieve a concentration ranging from 0.1 - 0.3 µg/ml in cerebrospinal fluid. Minimal inhibitory concentrations (MIC) for *S. neurona* are not available; however, a concentration of 0.2 µg/ml is required to inhibit *T. gondii*. A synergistic effect of the additional treatment with a sulfonamide was not investigated (Clarke 1992). Concurrent administration of dimethylsulfoxide (DMSO) (40% solution at 1g/kg bw) followed by an intravenous sulfamethoxazole-trimethoprim combination increased the intrathecal concentration of the drugs (Green 1990). Other pharmaceuticals such as diclazuril, a benzeneacetonitrile, and toltrazuril, a triazinon drug are under investigation (Tobin 1998, Furr personal comm.). Their mode of action is not known (Adams 1995).

Despite intensive treatment with sulfadiazine or sulfamethoxazole/trimethoprim in combination with pyrimethamine, the majority of the horses that presented for a repeat evaluation up to six month after initiation of treatment still remain positive on an immunoblot assay on CSF. Most of the horses however, show (good) clinical improvement (Fenger 1997). Drug resistance is a concern, that has been described when treating *Plasmodium falciparum*. Mutations of *P. falciparum* had a less susceptible dihydrofolate reductase and dihydropteroate synthase (Plowe 1997).

### **3 Central Nervous Inflammation and Immunity**

#### **A. Introduction**

Equine Protozoal Myeloencephalitis is an infection of the central nervous system, and therefore an infection of one of the so called 'immune privileged sites' of the body. The expression 'immune privileged site' (IPS) had been defined by Medawar and colleagues in 1948 (Medawar 1948). Their definition was based on findings of allogeneic graft acceptance when transplanted into the central nervous system, while the same grafts were vigorously rejected when transplanted to other sites of the body. Immune privileged tissue sites based on Medawar's definition are the gonads, the maternal-fetal interface, and the

central nervous system including the eye (Barker 1977). Central nervous inflammation due to an infectious organism depends on the type and number of organisms, and whether the organisms multiply within the CNS. The inflammatory response can be mild (edema, perivascular cuffing and gliosis) to severe and life-threatening (meningitis, demarcation with abscessation) (Summers 1995). The integrity of the central nervous system is crucial for body function. Its proper function depends on the integrity and interaction of neurons and their synapses. The absence of *de novo* synthesis of neurons after cell death is standing dogma, and functional immunosuppression within the CNS is a means to limit the extent of inflammation and increase the likelihood of return to normal function. This immunosuppression might sacrifice some effector mechanisms which allow pathogens such as *Herpes simplex Virus* and *Toxoplasma gondii* in humans to remain within the CNS as a latent or chronic persistent infection (Hunter 1994, Honing 1997). However, if an inflammatory insult is strong enough, the inflammatory effector phase response can be as strong and vigorous as at any other site of the body. Tissue injury results during which functional tissue is replaced by scar tissue unable to conduct impulses between the different areas of the brain, or towards the periphery. As Leslie Brent states “...It may be supposed that it is beneficial to the organism not to turn the anterior chamber or the cornea of the eye, or the brain, into an inflammatory battlefield, for the immunological response is sometimes more damaging than the antigen insult that provoked it...” (Brent 1990).

#### B. Cell populations of the CNS

The nervous system parenchyma consists largely of two cell populations: neurons and glial cells (see Table 1). Neuron function is restricted to conduction of electrical information, while glial cells have a supportive function. Glial cells differ from neurons in their lack of synaptic contacts, and they maintain the property of mitosis throughout life, and in particular as a response to injury (Raine 1994). Glial cells are subdivided into macro

and microglia. Microglia are of mesodermal origin and invade the CNS at the time of embryonic vascularization of the CNS.

Tab. 1: Functional classification of cells of the central nervous system

Cell type		Function
Glial Cells	Macroglia	
	-Oligodendrocyte -Schwann cell -Astrocyte	produces myelin in CNS produces myelin in PNV antigen presentation, neurotransmitter recycling antigen presentation, phagocytosis
	Microglia	
Neuron		conduction of electrical impulses

Microglia maintain mobility, which is essential in cases of inflammation. They migrate to areas of inflammation, and are involved in phagocytosis, antigen presentation, and the production and release of inflammatory mediators. The mediators have an auto, para or possibly endocrine function, which signal to other cell populations (lymphocytes, polymorphonuclear cells and astrocytes). Microglia are part of the reticulo-endothelial system (RES), and are related in origin, function and morphology to monocytes. It is thought that they form a resident population of cells of the RES within the CNS (Summers 1995, Hickey 1988). Whether there is a truly sequestered population of RES cells in the CNS, or whether they are continuously replaced by blood derived progenitors is not clearly understood (Keane 1997).

Macroglia consists of astrocytes and oligodendrocytes which are of ectodermal origin. Oligodendrocytes are responsible for the production of myelin within the CNS. The function of astrocytes is less well defined. They play an important part in antigen presentation, cytokine secretion, and they are part of the blood-brain barrier (Summers 1995, Keane 1997). Lymphocytes are virtually absent in normal CNS (Hickey 1991).

### C. Blood-Brain Barrier

The blood-brain barrier (BBB) is a functional and anatomical barrier between the CNS and the body. Access to the CNS is strictly regulated, and the barrier guarantees a microenvironment which keeps the CNS functional. The BBB is not a single tissue layer, however, is composed of two bordering tissue linings. The cerebral endothelial cells are on the peripheral side of the BBB. They form tight junctions between cells and a basement membrane. Foot-like processes of astrocytes line the inner side of the BBB, and they form a neuroglial membrane which lines the endothelium (fig. 5).

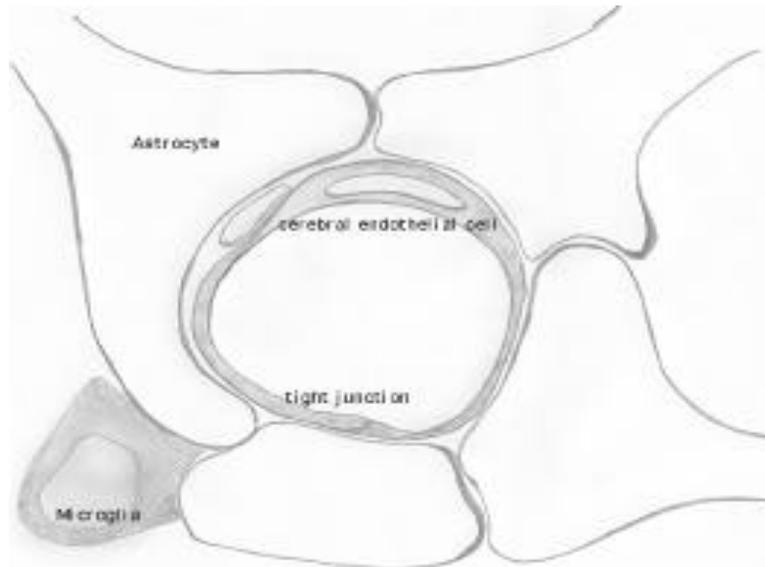


Fig. 5: Schematic drawing of the blood-brain barrier.

Distinct areas of the BBB differ in their morphology. Although the BBB is considered a closed membrane in healthy mammals, there is evidence of transport systems and membrane receptor interactions, following the release of second messenger molecules which are parts of a signaling system between the periphery and the CNS (Licinio 1997). Increased permeability of the BBB is a pathologic event, which allows access of plasma proteins, complement factors, and inflammatory cells into the CNS. This reflects as an increase in the albumin quotient (CSF albumin/ serum albumin) and pleocytosis of the CSF.

#### D. Immune response in the CNS

Medawar (1948) believed that the phenomenon of allogeneic graft acceptance seen at an immune privileged site (IPS) was attributed to the anatomical and functional separation between brain parenchyma and peripheral tissues. Experiments have shown however, that the administration of antigen intrathecally induces an antibody response in the periphery (Kaplan 1975). Thus, the antigen has to be brought in contact with the peripheral immune system. The graft acceptance within the CNS could therefore not be explained by sequestration of antigen. An immune response of the CNS is provoked after sequential steps of activation. First, the antigen has to enter the CNS, then the CNS has to activate the effector arm of the immune system, which results in entry of inflammatory cells into the CNS.

##### *i.* Antigen entry into CNS

Several routes are described for antigen access into the CNS. The entry mechanism is specific for the antigen. An axonal transport mechanism is described for the *Herpes simplex Virus*, where the virus invades a peripheral (cranial) nerve, and is transported retrograde towards the cell body of the neuron (Kristersson 1987). Other infectious organisms use cells on a routine surveillance mission as a carrier into the CNS (Trojan Horse hypothesis), and are transported passively and intracellularly into the CNS. *Listeria monocytogenes*, an intracellular pathogen is transported across the BBB by the macrophage (Keane 1997). The infection of cerebral endothelial cells is thought to be essential for the entry of *Sarcocystis neurona* and *Toxoplasma gondii* (Dubey 1989, 1994), however, the BBB is not damaged by this process.

##### *ii.* Activation of the effector arm of the immune system

Activation of the effector arm results in lymphocyte migration across the BBB into the CNS. Two different theories have been proposed for this activation process. Hickey (1991) proposed a generalized non-specific activation, while Cserr and Knopf (1997) proposed an antigen-specific activation of lymphocytes. Hickey et al. showed in 1991 that lymphocytes have to be activated before they enter the CNS. T-Lymphocyte entry was shown to be independent of antigen specificity, MHC-restriction, and T-cell phenotype. It is thought that possibly a soluble factor which is produced within the CNS causes generalized lymphocyte activation and causes migration of lymphocytes across the BBB. Hickey (1991) also demonstrated that once the activated lymphocyte exits within 24-48 hours, if it does not encounter its specific antigen. The other theory is based on an antigen specific activation, which is proposed to occur in cervical lymph nodes, the primary filter station of CSF (Cserr 1992, Cserr 1997). CSF leaves the CNS through three different routes: along the arachnoid villi into the dural sinus vasculature, along cranial nerves I, II, V, and VIII, and along spinal nerve roots. The cervical lymph nodes were identified as filter stations of the brain and the cervical spine, where antigen is presented to its specific lymphocytes. Perry and Gordon found a macrophage or dendritic cell population within the choroid plexus, which differed from microglia. These cells could function as true antigen presenting cells similar to Langerhans cells in the skin (Perry 1997). The antigen specific activation of lymphocytes, as proposed by Cserr, Knopf, Perry and Gordon requires that processed antigen leaves the CNS and consequently activates its antigen specific lymphocytes outside the CNS. Activation of lymphocytes results in production of IFN- $\gamma$  which is essential for the upregulation of specific adhesion molecules on the surface of cerebral endothelial cells. Adhesion molecules are under normal circumstances not expressed on the endothelium of immune privileged sites. In an *in vitro* study Male and coworkers (Male 1990b) showed that the exposure of

cerebral endothelial cells to FN- was necessary for the upregulation of lymphocyte adhesion molecules. In a multistep process, receptors and receptor ligands were expressed on the cell membrane of the leukocytes, the cerebral vascular endothelium, and the glial cells, in particular under the influence of IFN- (Dopp 1994).

Endothelial cells express two different classes of molecules for the entry mechanism of lymphocytes across the BBB: intercellular adhesion molecules type 1 (ICAM 1), and the vascular cell adhesion molecule (VCAM-1). The counterpart ligands on leukocytes are LFA-1 (ICAM1-LFA1), Mac-1 (ICAM1 - Mac1), and VLA-4 (VCAM1 - VLA4). LFA-1 and Mac-1 are expressed on the cell surface of respectively lymphocytes and macrophages. VLA-4 is a cell surface molecule on lymphocytes, macrophages and on some granulocytes. Fast upregulation (hours) and increase in number of ICAM is stimulated by FN- (Abbas 1994), while VCAM expression is delayed for several days. (Dopp 1994).

### iii. Antigen presentation

Astrocytes and microglia are antigen presenting cells (APCs) of the central nervous system. Both astrocytes and microglia have the ability to upregulate MHC II which is necessary for antigen recognition by the antigen specific lymphocytes. Secreted proinflammatory cytokines play a crucial role in the early process of antigen recognition. Once the T-Lymphocyte has entered the CNS it encounters the antigen presenting cells (APCs) of the CNS. Under the influence of FN- , Tumor Necrosis Factor- (TNF- ), and Interleukin-1b (IL-1b) astrocytes quickly express ICAM-1 mRNA, while ICAM-1 mRNA in microglia cells seems under influence of FN- only (Shrikant 1995). FN- is not produced by glial cells (astro nor

micro), and is solely produced by the T-cell. (Otero 1994). ICAM and VCAM are important co-stimulatory factors enhancing antigen recognition and propagation of an immune response. In a more recent publication Chabot showed that microglia produce TNF- $\alpha$  in a contact dependent manner. The ligands which were expressed were VLA-4 on the T-lymphocyte and VCAM-1 on the microglia cell (Chabot 1997).

iv. Pro-inflammatory immune response

At any other site of the body (any other 'non-privileged' site), recognition of specific antigen by the activated T-cells along with the presence of co-stimulatory molecules results in a clonal expansion and initiation of a T-helper 1 (TH1) or T-helper 2 cell (TH2) response. The dichotomy of T-helper cells is established for a variety of mammalian species, including the horse and bovine species (Swiderski 1998). The clonal expansion of TH1 results in a cell-mediated immune response, while a TH2 expansion induces a B-lymphocyte response with antibody production (Abbas 1994). A typical TH1 cytokine profile consists of IL-2, IFN- $\gamma$ , and TNF- $\alpha$ . Type 2 cytokines tend to support phagocyte independent mechanisms and are grouped as IL-4, IL-5, IL-10, IL-13 (Abbas 1994). The initial clonal expansion of T-cells is stimulated by autocrine IL-2 production (Otero 1995). Depending on the TH-subtype a characteristic profile of soluble cytokines is produced, which leads to B-cell activation and antibody production, stimulation of cytotoxic T-cells, macrophage activation, and depending on the stimulus, chemotaxis of polymorphonucleated cells. Cytokines are produced during the effector phase of natural and specific immunity and serve to mediate and regulate immune and inflammatory responses. Cytokine secretion is brief and self-limiting as some cytokines have an inhibitory function on others. Cytokines can act on a variety of cells, and they often have multiple effects on the same target cell. One response of the target cell is the synthesis and release of products which can be either

synergistic or antagonistic to immune cell function. Cytokines act on target cells by binding to a surface receptor, and can elicit an autocrine, paracrine or endocrine action (Abbas 1994). The inflammatory response is modulated by regulatory cytokines, which differ from the pro-inflammatory cytokines. IL-6 was recently characterized as a modulating cytokine (Xing 1998). Several studies showed the permanent expression of TGF- $\beta$  and IL-10 at IPS (see under), and these cytokines appear to have a significant role in CNS associated immune deviation in modulating the severity of the effector arm response.

#### E. Immune Deviation at Immune Privileged Sites (IPS)

Within the CNS, and with an intact BBB, upregulation of the effector arm of the immune system differs from other sites of the body. One strategy employed in immune-privileged sites is to create a local immunosuppressive microenvironment. This inhibitory milieu appears to be contributed to in part by immunosuppressive cytokines and substances present in biological fluids that bathe these sites. There are multiple mechanisms by which T-cell mediated processes can be suppressed. Responding lymphocytes can become functionally inactivated, or inactivated by initiating apoptotic cell death (Keane 1997). A range of soluble substances were identified, which have an *in vivo* and/or *in vitro* effect on the cells involved in defense mechanisms. Several substances with immunosuppressive properties were identified in brain tissue or in cerebrospinal fluid: alpha-Melanocyte Stimulating Hormone ( $\alpha$ -MSH), Vasoactive intestinal Peptide (VIP), Calcitonin Gene related Peptide (CGRP), Somatostatin, and the cytokines Transforming Growth Factor- $\beta$  (TGF- $\beta$ ), IL-4 and IL-10 (Miller 1992, Wilbanks 1992, Catania 1993, Taylor 1996a,b). A highly connected relationship is suspected among these factors, especially between TGF- $\beta$  and IL-10 (Keane 1997).

$\alpha$ -Melanocyte Stimulating Hormone

-MSH is a cleavage product of the pituitary proopiomelanocortin (POMC) peptide (Wilson 1982). Besides the pituitary gland it can be found in other regions of the brain, such as the hypothalamus, brain stem, and in the CSF. It is also found in other immune privileged sites such as the gonads and the placenta. It is a potent modulator of fever and the acute phase response, and seems to counteract the effects of TNF- $\alpha$ , IL-1 and IL-6 (Catania 1993). Taylor and Streilein identified  $\alpha$ -MSH in a low molecular weight fraction of human CSF, which inhibited T- cell proliferation in a dose dependent manner. They suggested that the role of  $\alpha$ -MSH is to reduce the production of IFN- $\gamma$  (Taylor 1996a).

#### Interleukin-10

IL-10 is produced by all three subsets of T helper cells (TH0, TH1, TH2), however, TH2 cell production predominates. To a limited extent it is also produced by monocytes, macrophages and B-cells. IL-10 inhibits macrophage antigen presenting functions, T-cell proliferation, and inhibits in particular cytokine synthesis by TH1 cells (Navikas 1996). Its concentration within CSF seems to increase during phases of remission of Multiple Sclerosis, and its concentration is decreased during an acute inflammatory phase of the disease. Together with cytokines produced by TH2-cells (IL-4, IL-5, IL-6, IL-13), it favors a differentiation of TH0 cells to TH2 cells, and suppresses the activity of existing TH1-clones. IFN- $\gamma$  concentrations as a cytokine produced by TH1 are significantly decreased in the presence of high concentrations of IL-10. TH2 cells control the humoral immunity (production of antibodies), participate in the extracellular defense against protozoa, and cause allergy (Navikas 1996).

#### Transforming Growth Factor- $\beta$ (TGF- $\beta$ )

TGF- $\beta$  occurs in 5 different subtypes. Only type 1 through 3 are found in mammals; isotype 2 and 3 are only found in fetuses. TGF- $\beta$  1 is a homodimeric protein that is synthesized in its latent form, and has to be activated by proteases. TGF- $\beta$  is a potent

immunoregulator. It inhibits T- and B-cell proliferation, thymocyte proliferation, natural killer cell activity, and cytotoxic killer cell activity (Flaumenhaft 1993). In the CNS there is a permanent, measurable concentration of TGF- $\beta$ , which increases during disease and trauma (Keane 1997). TGF- $\beta$  has a suppressive effect on the effector arm of the CNS immune system. It interferes with antigen presentation and clonal expansion. It suppresses the expression of adhesion molecules and MHC II on astrocytes, decreases the proliferation of astrocytes, and it stimulates oligodendrocyte/Schwann cells to proliferate, which is important for myelin production during repair (Otero 1994).

Wilbanks demonstrated that macrophages from the peritoneal cavity could be endowed with the capacity to induce an antigen-specific immune deviation by co-culturing these cells with either CSF or aqueous humor. By adding anti-TGF- $\beta$ -antibodies to the CSF/aqueous humor he showed a complete restoration of the macrophage function (Wilbanks 1992). He postulated that TGF- $\beta$  is continuously expressed within the CSF, and it is responsible for the immune response deviation that is described at IPS. Taylor and coworkers (1996a, b) performed a lymphocyte blastogenesis assay in which cells were coincubated with CSF. Cells coincubated with CSF had a decreased production of IFN- $\gamma$  when compared to control populations. Acidification of the CSF enhanced the immunosuppressive effect, as demonstrated by TGF- $\beta$  activation. Whole CSF did not have as strong a suppressive effect as fractionated CSF. Because the activation of latent TGF- $\beta$  requires proteolytic cleavage, which can be simulated by acidification of the sample, Taylor concluded that most of the TGF- $\beta$  in the CSF is in its latent form. Taylor attributed the immunosuppressive effect he saw in the small molecular weight fraction in part to active TGF- $\beta$ . However, he could not exclude other factors with an effect on lymphoblastogenesis such as  $\alpha$ -MSH, VIP, and others (Taylor 1996).

#### F. Central nervous immunosuppression and protozoal infections

Protozoan infections are common in immunocompromised individuals, and are therefore frequently described as opportunistic infections. A few protozoan organisms such as *Neospora spp.*, *Toxoplasma spp.*, and *Plasmodium spp.* are known to invade the central nervous system. While the CNS can become infected during immunosuppression of the host, some pathogens are able to enter the CNS of a host without pre-existing immunosuppression. *Toxoplasma gondii* cysts are commonly found in the CNS of humans, where they remain in a dormant stage, possibly life-long. *Toxoplasma* serum antibodies are detected in 3 - 45% of the adult population of the United States. *Toxoplasma* cysts within the CNS are found in 80% of the seropositive cases (Hunter 1994). If immunosuppression occurs in the host due to an immunodeficiency disease such as AIDS, or immunodeficiency is induced during organ transplantation or chemotherapy, the CNS cysts of *T. gondii* can become activated and resume their phase of replication. This re-activation of dormant cysts during immunosuppression causes toxoplasmic encephalitis (TE). Multiple brain abscesses are the most common feature of TE in AIDS patients. Histologically the abscesses are characterized by an avascular central zone containing necrotic material. Surrounding this area is a hyperemic region containing numerous free parasites and parasitized cells as well as infiltrating mononuclear cells (Hunter 1994). Because of the frequency and severity of these complications toxoplasmosis and TE are currently being vigorously investigated and reviewed (Hunter 1994, Hunter 1996, Nagasawa 1996, Däubner 1997, Innes 1997).

Based on phylogenetic and morphologic similarities, and some pathophysiologic mechanisms *T. gondii* encephalitis may be a valuable model for investigation of *S. neurona* interaction in the equine nervous system. As mentioned in Section 2C (Life cycle of *S. neurona*) the differences between the two protozoan organisms lie within their reproductive cycle and morphology. Of importance is that a) *Toxoplasma* develops tissue cysts within the CNS, a feature that has not been described in horses with EPM, and b) TE only develops in immunocompromised individuals. The immune status of EPM affected horses

is not adequately well characterized to support such a statement, however, EPM does not occur more frequently in horses in conjunction with other opportunistic infections.

Epidemiological studies regarding the occurrence of EPM did not identify risk factors associated with the immune status of the horse (Bentz 1997, Blythe 1997, Saville 1997).

Two groups of soluble cytokines play an important role in the defense mechanism in *T. gondii*. IFN- $\gamma$  is necessary for protozoan intracellular killing (Hunter 1994), and this killing mechanism is counteracted by increased concentrations of IL-10 and TGF- $\beta$ . An imbalance between IFN- $\gamma$  and TGF- $\beta$  / IL-10 may cause a decreased production of oxygen free radicals and reactive nitrogen intermediates. Both are believed to be key to the protozoan killing mechanism on a molecular level. Their concentration increases under influence of IFN- $\gamma$ , and decreases with increasing amounts of TGF- $\beta$  / IL-10 (Hunter 1994). As discussed in 2E (Immune deviation at immune privileged sites) the constitutive expression of IL-10 and TGF- $\beta$  at IPS may create a microenvironment for the protozoan organism that enhances its survival by counteracting the killing mechanism directed against the protozoan..

Two studies further complement the importance of TGF- $\beta$  in the pathophysiology of protozoan-host interactions. Ming (1995) demonstrated an enhanced cell invasion of *Trypanosoma cruzi* when non-phagocytic cells were pretreated with TGF- $\beta$ . This effect, however, was not seen with *T. gondii*. Ming proposed that *T. cruzi* itself triggers the activation of the TGF- $\beta$  signaling pathway which is required for the parasite entry into the mammalian cells (Ming 1995). The second study (Barral-Netto 1992) showed that *Leishmania spp.* induces the production of TGF- $\beta$  in vitro and in vivo which is necessary for the survival of protozoan stages within the infected macrophages.

Very little is known about the immune system interactions of *S. neurona* which causes myeloencephalitis in horses. A recent publication showed the in vitro immunogenicity of two surface molecules of *S. neurona* (Sn14 and Sn16), which caused

an antibody response and which are potentially important for a defense mechanism against extracellular stages of the protozoon (Liang 1998).

## Conclusions

Equine Protozoal Myeloencephalitis is a relatively new disease entity, and the body of knowledge accumulated so far does not correlate with the extent of the disease. Since the introduction of the Western Blot and PCR for EPM major achievements have been made in epidemiology and in unraveling the protozoon's reproductive cycle, which includes the definition of its definitive and intermediate hosts. There is a lack of knowledge regarding the interaction of *S. neurona* and the equine immune system. It is unknown whether the microenvironment of the CNS provides an immunosuppressive milieu to diminish or decrease active inflammation within a tissue site with a poor repair capacity.

Pathogens such as *T. gondii* benefit from the environment at immune privileged sites. The effector arm is less effective due to an imbalance between the concentrations of IFN- and TGF- / IL-10. Whether this is the result of a TH2 cell predominance is not known. For pathogens such as *T. gondii* there seems to be a life-long presence within the central nervous system of the host. Although the persistent production/presence of intrathecally produced antibodies against *S. neurona* could be the result of continuous immunogenicity of protozoal remnants, it is possible that there is a chronic persistent infection with *S. neurona*.

Protozoa might need a TGF- signaling pathway to penetrate cells of the CNS. They might induce TGF- production or they may benefit from constitutively expressed amounts of TGF- . However, this TGF- dependent effect has only been shown in some *Trypanosoma spp.* and *Leishmania spp.* This effect could not be demonstrated in *T. gondii*, which may or may not be relevant for *S. neurona*.



## **Hypothesis:**

1. We hypothesize that *Sarcocystis neurona* causes immunosuppression during cell growth.
2. We propose that the central nervous system of horses maintains an immunosuppressive microenvironment. This microenvironment might facilitate access of pathogens into the CNS, and also facilitates the persistent presence of the pathogens.

Therefore, two experiments were designed. In Experiment one we attempted to determine whether fragments of *S. neurona* decrease the *in vitro* proliferative ability of lectin stimulated lymphocytes. In Experiment two the objective was to detect a decrease in proliferative ability of lectin stimulated lymphocytes when co-incubated with equine CSF.

## **4. General Material and Methods:**

Horses affected with Equine Protozoal Myeloencephalitis (EPM) were selected by two criteria. They exhibited clinical signs of EPM, and specific intrathecal antibodies were detected in cerebrospinal fluid by immunoblot analysis (Western blot). EPM horses were euthanized at the end of the experimental phase and the diagnosis of EPM was confirmed by gross and histologic examination. The control group of horses comprised animals that did not show clinical signs of neurologic disease, and specific antibodies against *S. neurona* were absent in serum and CSF, as detected by immunoblot analysis (Western blot).

### **Experiment 1**

Peripheral blood mononuclear cells (PBMCs) from EPM horses and control animals were stimulated with a submaximal concentration of a T cell specific mitogen, concanavalin A. PBMCs were co-cultured with cellular fragments of *Sarcocystis neurona* - merozoites, and the proliferation was assessed by using a <sup>3</sup>H-Thymidine incorporation assay. The blastogenic cells incorporate the radioactive labeled thymidine into their DNA. Excessive, non-incorporated <sup>3</sup>H-Thymidine is removed during a filtration procedure, and emission of beta-radiation was measured in the sample. To address the possibility that proliferation was limited due to an increase in cell death of cells in culture, cell viability was assessed with a routine Trypan Blue staining technique.

### **Experiment 2**

Peripheral blood mononuclear cells from an EPM horse and a control were stimulated with a submaximal dose of a T-cell specific mitogen (concanavalin A). Cerebrospinal fluid (CSF) from EPM horses and controls was used as a substrate for co-

culture during the proliferation assay. The CSF was processed further prior to co-culture. CSF was filtered through 2 microfilter units with different pore size. This process resulted in three CSF fractions, which differed in protein concentrations and molecular size. Each of the three fractions was used as a co-culture substrate in the proliferation assay. An identical volume of saline (NaCl 0.9%) was used in a separate assay to correct for a possible effect caused by dilution of media. PBMC proliferation was assessed by a  $^3\text{H}$ -Thymidine incorporation assay identical to the procedure described in experiment 1. Beta emission was measured and the results were compared to the saline control. Results were also compared between the cell donors (EPM vs. control). As for experiment 1 viability of cells in co-culture with the CSF fractions was assessed by a Trypan Blue<sup>TM</sup> staining technique, followed by a manual cell count to exclude a toxic effect of the fractions on the lymphocytes in culture. All EPM horses were euthanized after completion of the experiments. A post mortem exam of the central nervous system was done to confirm the diagnosis of EPM.

## 5. Experiment 1

Title: Effect of Sarcocystis neurona -lysates on mitogen-induced proliferation of equine lymphocytes: A comparison between Horses affected with Equine Protozoal Myeloencephalitis (EPM) and control horses.

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**Abstract:** Equine Protozoal Myeloencephalitis (EPM), caused by the protozoan Sarcocystis neurona is currently the most frequently diagnosed disease of the central nervous system (CNS) in horses in the United States. Despite a prolonged therapeutic treatment elimination of the infection is difficult. This fact is supported by the persistent presence of S. neurona specific intrathecal antibodies reflecting antigenic stimulation, and the possible reoccurrence of disease-specific clinical signs after discontinuing medication. Impairment of the immune response is suspected, which could be caused by two different mechanisms. The CNS as an immune privileged site with a possibly immunosuppressive environment could enhance survival of the protozoan organism. A second mechanism could involve the production and release of an immunosuppressive substance directly by the pathogen, which could also enhance its chance of survival at the infection site. In this study, we investigated the influence of the protozoan organism on the host's immune response, and in particular the effect of Sarcocystis neurona -infected bovine turbinate cells on proliferation of mitogen-stimulated peripheral blood mononuclear cells (PBMCs) from horses. PBMCs from 8 EPM and 5 control horses were co-cultured with fragments from S. neurona merozoites and the effect on concanavalin A-induced lymphocyte proliferation was determined. Lymphocyte proliferation was significantly decreased (p 0.05) when PBMCs were co-cultured with S. neurona merozoites. There was no decrease in viability of lymphocytes cultured with S. neurona, suggesting that the substrate did not cause an

actual decrease in cells participating in this assay. These results suggest that the persistence of S. neurona infection in the horse's CNS is, in part, due to a pathogen-derived mechanism that affects the host's immune response.

### **Introduction:**

Equine Protozoal Myeloencephalitis (EPM) is the most frequently diagnosed disease of the central nervous system (CNS) of horses in the United States and is caused by a protozoan organism of the genus Sarcocystis (sp.: S. neurona). The horse is not the natural intermediate host, but an aberrant host, and becomes infected by ingestion of infectious sporocysts. Sporocysts originate from the intestinal tract of the definitive host, the opossum (Didelphis virginiana), where S. neurona completes its sexual phase of reproduction. The site of infection in the horse is atypical for Sarcocystis spp. The protozoan organism enters the CNS of horses, and induces lesions during invasion and replication. The most common presenting clinical sign is an asymmetric ataxia of the limbs, however, clinical signs can vary based on location and number of lesions (MacKay 1997). Presence of intrathecal antibodies as detected by western blot (WB) is used as a diagnostic aid in combination with clinical signs. Intrathecal production of antibodies is assured by evaluation of the blood-brain barrier integrity as evaluated by albumin quotient and IgG-index (Andrews 1997). A PCR was developed, however, sensitivity of this test was shown to be low and has since fallen out of favor (Reed 1996). Blot densitometry is being investigated for its use to distinguish intrathecal antibody production from the diffusion of blood derived specific antibodies into the CNS (Knopf 1998, Fenger 1998b). The current recommended treatment is a combination of sulfonamide antibiotics and pyrimethamine for an average duration of six months. Western blot analysis is also used to monitor response to therapy. Absence of measurable intrathecal antibodies is considered representative of resolution of CNS infection. Remission of clinical signs however, typically precedes negative WB analysis. Further, some clinically normal animals continue to maintain measurable antibody concentrations even after prolonged treatment (12 months) (Fenger

1998a). The need for prolonged treatment suggests that elimination of the Sarcocystis infection by the equine immune system is at best inefficient. There are two possible explanations for this observation. The CNS is considered as an immune privileged site, where the host response is attenuated in order to prevent excessive tissue destruction (Barker 1977). It is also possible that the pathogen, Sarcocystis neurona, induces an immune suppressive effect, analogous to other protozoan organisms (Ming 1995).

T lymphocytes play a predominant role in the elimination of protozoan organisms (Abbas 1994). The goal of this study was to evaluate the potential immunosuppressive effects of Sarcocystis neurona infection on the function of equine lymphocytes *in vitro*. To accomplish this, peripheral blood mononuclear cells (PBMCs) were collected from EPM-affected and control horses. The *in vitro* response to mitogenic stimulation with concanavalin A (ConA) was measured when cells were co-cultured with lysates of S. neurona merozoites, or control substrates that lacked S. neurona-derived components. PBMCs, co-cultured with S. neurona merozoite- lysate demonstrated a significant decrease in mitogen-induced proliferation, when compared to the control substrates. These results suggest that the persistence of S. neurona infection in the horse's CNS is, in part, due to a pathogen derived mechanism, which either directly or indirectly may hinder host immune response.

### **Material & Methods:**

#### *Horses:*

Thirteen horses of various breeds were used in this study. The age distribution was between 2 and 20 years. EPM was diagnosed by characteristic clinical signs of the disease in conjunction with the presence of intrathecal antibodies as detected by Western blot analysis (Equine Biodiagnostics, Lexington, KY) of a cerebrospinal fluid sample (Granstrom 1995). Seven out of 8 EPM horses were euthanized at the end of the experimental period. The presence of typical gross pathologic and histologic findings of the

central nervous system further substantiated the EPM diagnosis (Mayhew 1978). Control horses were selected based on a negative neurologic exam by two independent clinicians, and negative immunoblot (WB) results on serum and CSF. The gender distribution of the 8 EPM horses was 5 geldings and 3 mares, and control horses was 2 geldings and 3 mares. Horses were maintained on pasture, and diets were supplemented with hay and water. All maintenance and care were in accordance with the animal welfare guidelines established at the Virginia Polytechnic Institute and State University.

*Isolation of Peripheral Blood Mononuclear Cells (PBMCs):*

PBMCs were isolated using a modification of previously described methods (Ansar Ahmed 1993). Approximately 40 ml of peripheral venous blood was collected into heparinized tubes (Vacutainer™, Becton Dickinson, Franklin Lakes, NJ) by aseptic venipuncture from the left or right jugular vein from control and EPM-affected horses. Routine leukocyte counts (CBC) and leukocyte differentials were performed from separate EDTA- blood. The heparinized blood was centrifuged first at 600 x g for 10 minutes. The buffy coat was recovered and re-suspended collectively in 8 ml of RPMI 1640 incomplete medium. One ml of the cell suspension (approximately  $20 \times 10^6$  leukocytes) was placed on top of 3 ml of ficoll-hypaque (Lymphoprep™, Nycomed Pharma AS, Oslo, Norway) in sterile 15 ml centrifuge tubes at room temperature. Separation of lymphocytes was achieved by continuous gradient centrifugation at 350 x g, 30 minutes at 20°C. Cells were recovered at the ficoll-hypaque cell suspension interface by gentle aspiration, and re-suspended in incomplete RPMI 1640 medium. The cells were washed three times, and finally stored in complete RPMI 1640 medium (Cellgro™ RPMI 1640 1x (Mod.) with L-glutamine, HEPES Buffer 25 mM, 10% heat inactivated fetal bovine serum, and penicillin (50 IU/ml)-streptomycin (50 µg/ml)) on ice for no more than one hour. Following cell count with hemacytometer and cytologic evaluation, cell suspensions were diluted with complete medium to a final concentration of  $5 \times 10^6$  mononucleated cells/ml.

*Preparation of Co-culture Supernatants and Lysates:*

The co-culture substrates were provided by Dr. D. Granstrom (Gluck Equine Research Center, University of Kentucky) using the following methods. Sarcocystis neurona -merozoites (SN3) were originally isolated from the spinal cord of a horse with histologically confirmed EPM, and were subsequently cultured in bovine turbinate cells, suspended in an RPMI 1640 medium (Granstrom 1992). For this experiment four different co-culture supernatants or lysates were provided:

- i) complete RPMI 1640 medium (Cellgro™ RPMI 1640 1x (Mod.) with L-glutamine, Hepes Buffer 25 mM, 10% heat inactivated fetal bovine serum, and penicillin (120 IU/ml)-streptomycin (120 µg/ml)), as prepared by the Gluck Equine Research Center.
- ii) cell-free supernatant from alive, non-infected bovine turbinate cells
- iii) freeze-thawed, non-infected bovine turbinate cells (non-infected cell lysate)
- iv) freeze-thawed bovine turbinate cells, infected with Sarcocystis neurona -merozoites (infected cell lysate).

All samples were frozen in 20 ml aliquots prior to shipping, thawed on arrival at the CMMID, Virginia Polytechnic Institute and State University in Blacksburg, and re-frozen in 2 ml aliquots until use.

*Lymphocyte Proliferation Assay:*

Equine lymphocytes ( $5.0 \times 10^6$  cells/ml) were cultured together with a fraction of one of the above four different culture supernatant or lysates. All four media-lysate or media-supernatant preparations included complete RPMI 1640 medium (890 µl) per 1000 µl volume. Additional volumes of concanavalin A (10µl (0.5 µg/µl)), and 100 µl of one of the four co-culture substrates were added to achieve a volume of 1000 µl. To a 96-well round bottom plate (Corning Glass Works, Corning NY) 100 µl of the cell suspension ( $5 \times 10^5$  mononuclear cells) from the EPM horse and the control horse were added. The cells

were incubated with 100 µl of the media preparations containing supernatant or different lysates which were added to quadruplicate wells, and incubated at 37°C (5% CO<sub>2</sub>). After 48 hours of incubation, the samples were pulsed with 1 µCi/well of <sup>3</sup>H-Thymidine, and incubated at 37°C (5% CO<sub>2</sub>) for an additional 18 - 20 hours. The cells were harvested with a PHD Cell Harvester (Cambridge Technology Inc., Cambridge MA.) over microfiber filters (Whitman Glass Microfiber Filters), placed in scintillation vials (Wheaton Scientific, Millville NJ), and mixed with 3 ml scintillation fluid (Ecoscint™, National Diagnostics, Atlanta GA). The vials were placed in a Beckman LS 8100 scintillation counter, where beta-emission was counted for ten minutes, and results were expressed in counts per minute (CPM). The mean and standard error of mean (SEM) were calculated for each quadruplicate.

*Analysis of Lymphocyte Proliferation Assay:*

The proliferation data from cells co-cultured with complete RPMI 1640 medium (Gluck Equine Research Center) served as control for comparison with cells co-cultured with supernatant (uninfected cells), and lysates from uninfected and infected cells. The mitogen-induced lymphocyte proliferative response in complete RPMI 1640 medium (containing media only from the Gluck Equine Research Center as a co-culture substrate) was considered the standard (100%). The CPMs obtained from culture with supernatant and lysates from uninfected and infected turbinates were divided by the standard results in CPMs, multiplied by 100, and expressed as a percentage change .

Separate calculations compared the results obtained from PBMCs cultured with cell fragments from uninfected and infected turbinates. This comparison was done because the composition of cell fragments from uninfected turbinates was considered most similar to the infected cell fragments with the exception of *S. neurona* merozoite components. CPMs obtained from cell fragments from infected turbinates were divided

by the CPMs of the cell fragments from uninfected cells, and multiplied by 100 to determine a percentage.

*Viability assessment of the cells in culture:*

To address the possibility that proliferation was inhibited due to an increase in cell death, cell viability was assessed by a routine Trypan Blue exclusion (Trypan Blue 0.4%, Sigma-Aldrich, St. Louis, MO) staining technique after 24, 48 and 72 hours of incubation. Blood was obtained from 5 randomly selected horses. PBMCs were isolated by the previously described separation method and co-culture with three of the co-culture media, which were complete RPMI 1640 medium (Gluck Equine Research Center), lysates from uninfected and infected bovine turbinate cells, as described above. Number of cells per well, and volumes of substrates were identical to the proliferation assay. Co-culture with the supernatant of uninfected turbinate cells was not possible, since all of the original sample had been used in previous experiments. The percentage of dead cells was calculated, and compared between groups.

*Statistical Analysis:*

A paired Student's t-test was applied for statistical evaluation to determine differences between co-culture with media (100%), and the other co-culture substrates. A Wilcoxon ranked signed test was used to compare the results from co-culture with lysate from uninfected and from infected bovine turbinate cells. Standard desktop computerized statistical programs were used (Minitab Inc, State College, PA). Differences between groups were considered significant when  $p < 0.05$ .

**Results:**

*Lymphocyte Isolation and Recovery:*

All horses, which served as lymphocyte-donors, had a normal CBC on the day of the experiment. The white blood cell differentiation was normal except for a mild eosinophilia (5%) in one of the control horses. The eosinophilia of this particular horse did not appear to affect the mitogen-stimulated lymphocyte proliferation assay with different concentrations of Con A. General lymphocyte recovery ranged between 40 and 55%. Cytologic evaluation revealed a purity of more than 95% mononucleated cells, with about 80 - 90% lymphocytes. A small to moderate number of platelets was seen in the samples.

*Lymphocyte Proliferation Assay:*

The results of the proliferation assay with PBMCs co-cultured with the four co-culture substrates are summarized in Table 1. For both groups of horses (EPM vs. control) the mean proliferative response of cells co-cultured with cell fragments from infected turbinate cells showed a moderate but consistent decrease in proliferation, when compared to the results of the proliferation with media alone (Table 2). The proliferative response of cells co-cultured with cell fragments from infected turbinate cells were not significantly different between EPM horses and controls ( $p > 0.05$ ). There was a noticeable variation in individual proliferative responses among PBMCs isolated from EPM-affected horses. The greatest variation of results was seen in the results of PBMC proliferation co-cultured with the cell fragments from non-infected turbinate cells (Table 1). Five out of 8 horses in the EPM group showed an actual increase in PBMC proliferation in co-culture with the cell fragments from non-infected turbinate cells. Applying the Wilcoxon ranked sign test, which was used to evaluate differences between the results from non-infected and infected cell-lysates, a statistical difference ( $p < 0.05$ ) was found for the results obtained from EPM-affected horses. Proliferation decreased to  $(77.4\% \pm 5.3^*)$  when PBMCs were co-cultured with the infected cell lysate, compared to the non-infected cell lysate. The decrease in proliferation of PBMCs from control horses, when co-cultured with infected cell lysate was not significant, compared to the uninfected cell lysate  $(87.3\% \pm 3.7)$  (Table 2).

### *Viability Assessment:*

The viability staining of PBMCs co-cultured with lysates from *S. neurona*-infected or uninfected bovine turbinate cells or complete RPMI 1640 medium co-cultured with complete RPMI 1640 medium (Gluck Equine Research Center) with the Trypan Blue stain did not show an increased percentage of non-viable cells ( $p>0.05$ ) (Figure 1).

### **Discussion**

Currently, there is limited information about the host-protozoan interaction in EPM. It is suspected that the CNS microenvironment, or elaboration of immunoregulatory substrates from the pathogen itself contributes to the slow recovery of affected horses. This *in vitro* study evaluated one aspect of the pathogen-host interaction. Our findings suggest that the presence of merozoite stages of *S. neurona* has a negative effect on mitogen-stimulated lymphocyte proliferation.

The reduction in proliferation of mitogen-stimulated lymphocytes potentially could be caused by one of the following mechanisms. An infected cell, or *S. neurona* could produce one or more soluble factors that are able to decrease the rate of proliferation. The production of a soluble factor is supported by two studies involving Transforming Growth Factor- (TGF- ) in specific protozoan infections (Barral-Netto 1992, Ming 1995). Another possibility is, that cell wall fragments from either *S. neurona* merozoites, or from the infected cell potentially decrease lymphocyte proliferation. Lipopolysaccharides (LPS), a B lymphocyte mitogen (Abbas 1994), is an example of an interaction between cell wall-derived components and lymphocytes in culture. Alternatively, it is possible that an inhibitory effect on lymphocyte proliferation could be due to released intracellular substances (metabolites, cell organelles, lysosomes) of ruptured cells, either from *S. neurona* or the infected cell. Regardless of the source of an inhibitory factor or mechanism,

none of the co-culture lysates or supernatant promoted lymphocyte cytotoxicity, based on the trypan blue exclusion staining.

The use of supernatant from S. neurona-infected bovine turbinate cells in culture would have possibly demonstrated the presence of 'soluble factors'. To further evaluate the presence of a 'soluble factor' either produced by S. neurona or the infected cell a more appropriate substrate would have been the supernatant of S. neurona infected bovine turbinate cells in culture. This substrate would have allowed us to exclude an interference of lymphocyte proliferation by intracellular substances or cell wall fragments. However, the *in vitro* reproductive cycle of S. neurona (endopolygeny) in bovine turbinate cells is of relative short duration (approximately 36 - 48 hours). Merozoite stages liberate quickly into the medium and cause contamination of the supernatant with cell wall fragments and intracellular substances from the ruptured bovine turbinate cells. It was therefore not possible to obtain a sample that was free of ruptured host cells, and/or containing free *S. neurona* merozoites (D. Granstrom, pers. comm.).

Despite the possible inhibitory effect that cell wall fragments or intracellular substances might have on a lymphocyte proliferation assay, there is supportive evidence that certain protozoan organisms use a pathway for cell entry that includes induction of Transforming Growth Factor- (TGF- ). Leishmania spp. infections induce the production of TGF- (Barral-Netto 1992), and Trypanosoma spp. invasion of mammalian cells requires activation of the TGF- signaling pathway. Further, blocking TGF- with anti-TGF- monoclonal antibodies prevented the entry of the organism into the cells (Ming 1995). However, the TGF- dependent pathway as described for Trypanosoma spp. seemed not essential for Toxoplasma spp (Ming 1995). Toxoplasma spp., on the other hand, is closely related to Sarcocystis spp (Dubey 1989), and therefore, S. neurona may or may not use TGF- -dependent pathways. We expand this discussion about TGF- because of another important reason. This molecule is permanently expressed at immune privileged sites such as the central nervous system (Wilbanks 1992, Letterio 1997). Together with

other cytokines, TGF- $\beta$  regulates the microenvironment in the CNS to diminish an excessive inflammatory response. Another mechanism by which TGF- $\beta$  can regulate immune responses is by favoring TH 2 response instead of IFN- $\gamma$  producing TH 1 cell expansion (Horohov 1998, Swiderski 1998). Numerous studies have shown the importance of IFN- $\gamma$  in intracellular host defense such as in protozoan infections with Toxoplasma gondii, Leishmania spp., and Trypanosoma spp. (Hunter 1994, Alexander 1997, Däubener 1997).

This *in vitro* model provides preliminary information concerning a pathogen induced mechanism of local immunosuppression in the pathogenesis of EPM. This mechanism might be only of partial influence. It is important to point out that our studies dealt with lymphoproliferative responses induced by Con A, rather than *S. neurona*-derived antigens. It is certainly possible that *S. neurona*-derived antigen-specific lymphoproliferative response may simulate Con A, or may be normal or exaggerated. Thirty years of EPM research has yet to show protozoan replication in any organ system other than the CNS. Therefore it seems necessary to elucidate the protozoan-host interaction with emphasis on the CNS. Future studies will need to focus on the molecular pathway of decreased proliferation in mitogen stimulated lymphocytes in co-culture with *S. neurona* by electrophoresis and separation of protein fractions.

### **Acknowledgments:**

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Table 1: Individual results from mitogen-induced (ConA) lymphocyte proliferation assay in co-culture with: i) complete RPMI 1640 medium (Gluck Equine Research Center); ii) supernatant from alive, uninfected bovine turbinate cells; iii) cell fragments from uninfected bovine turbinate cells; iv) cell fragments from *S. neurona* -infected bovine turbinate cells. Results are grouped by horses with EPM and control horses. Results are expressed as a difference in percent (%) from the medium control (Medium = 100%). Results are also expressed as **Mean ± SEM**. Statistical significance (p 0.05) is indicated by \*).

	Complete RPMI 1640 medium (Gluck Equine Research Center)	Supernatant from uninfected bovine turbinate cells	Cell fragments from uninfected bovine turbinate cells	Cell fragments from bovine turbinate cells, infected with <i>S. neurona</i>
<b>EPM:</b>				
Horse 1	100	77.5	113	94.2
Horse 2	100	60.7	66.5	55.7
Horse 3	100	91.4	127	88.3
Horse 4	100	92.7	91.8	90.8
Horse 5	100	134	174	83.4
Horse 6	100	86.2	83.3	69
Horse 7	100	99.9	118	73.9
Horse 8	100	117	139	96.4
n=8		<b>94.9 ± 8</b>	<b>114 ± 12</b>	<b>81.5 ± 5*)</b>
<b>Control:</b>				
Horse 1	100	106	117	91.2
Horse 2	100	88.3	95.1	80.6
Horse 3	100	67.5	78.8	64.8
Horse 4	100	98.2	94.2	89.6
Horse 5	100	94.6	82.7	80.0
n=5		<b>90.9 ± 7</b>	<b>93.6 ± 7</b>	<b>81.2 ± 5*)</b>

Table 2: Statistical evaluation (p-value) of results. PBMCs from EPM affected and control horses were co-cultured with supernatant from uninfected bovine turbinate cells, or with a cell lysate from either uninfected or infected bovine turbinate cells. A Student's t test analyzed differences between the co-culture substrates compared to media alone. A Wilcoxon ranked sign test analyzed differences between results from uninfected cell lysate and infected cell lysate. \*) indicates statistical significance, which was pre-set at p 0.05.

<b>Statistical analysis:</b>	<b>PBMCs from EPM-affected horses (n=8)</b>	<b>PBMCs from control horses (n=5)</b>
Student's t test:		
Hypothesis: Proliferation of PBMCs with co-culture substrate is different from culture in RPMI 1640 alone.		
RPMI 1640- supernatant	p = 0.6	p = 0.24
RPMI 1640- uninfected cell lysate	p = 0.28	p = 0.4
RPMI 1640- infected cell lysate	p = 0.008 *)	p = 0.002 *)
Wilcoxon ranked sign test:		
Hypothesis: Proliferation of PBMCs, co-cultured with infected cell lysate is different from co-culture with uninfected lysate.		
infected cell lysate - uninfected cell lysate	p = 0.014 *)	p = 0.059

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## 6. Experiment 2

Effect of Cerebrospinal Fluid from Horses with Equine Protozoal Myeloencephalitis on Equine Lymphocyte Proliferation

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**Abstract:** Equine Protozoal Myeloencephalitis (EPM), which is caused by the protozoan Sarcocystis neurona (*S. neurona*) is currently the most frequently diagnosed disease of the central nervous system (CNS) in horses in the United States. Despite prolonged treatment elimination of the infectious agent cannot be guaranteed, as evidenced by the persistent presence of *S. neurona* specific intrathecal antibodies. Impairment of the immune response is suspected, which could be caused by two different mechanisms. The CNS as an immune privileged site with its immunosuppressive microenvironment could enhance survival of the protozoan organism. A second mechanism could involve the production and release of an immunosuppressive substance directly by the pathogen, which also would enhance its chance of survival at the infection site. In this study we investigated the effect of cerebrospinal fluid (CSF) on mitogen-stimulated peripheral blood mononuclear cells (PBMCs) from an EPM affected and a control horse. CSF from 11 EPM affected and 3 control horses was divided in three fractions after processing through microfilter units. All three fractions of the CSF from EPM affected horses caused significant decrease ( $p < 0.05$ ) in proliferation, when PBMCs from the EPM affected horse were used. CSF fractions from the control horses showed a similar trend. The response of PBMCs from the control horse was less pronounced in co-culture with the low-weight CSF fractions from EPM affected and control horses. The fraction containing molecules  $>10$  kD showed a statistically significant increase when cultured with mitogen-stimulated PBMCs from control horses. The decrease in lymphocyte proliferation is due to an effect derived from CSF, which did not cause increased cell-death. These results suggest that soluble factors, in part, have an influence on immune mediate processes in the CNS of horses, that attenuate the host's immune response.

## **Introduction:**

The immune privilege of the CNS provides a mechanism that prevents excessive tissue destruction during inflammation (Barker 1977). An immunosuppressive microenvironment is maintained by the blood-brain barrier (Brent 1990), persistent expression of Fas ligand (Abbas 1994), and by substances such as  $\alpha$ -Melanocyte Stimulating Hormone ( $\alpha$ -MSH), Vasoactive Intestinal Peptide (VIP), Interleukin-10 (IL-10), Transforming Growth Factor- $\beta$  (TGF- $\beta$ ), and probably others. These protein molecules cause a down regulation of adhesion molecules on cerebral vasculature and on glial cells, and therefore limit lymphocyte access into the CNS and antigen presentation of astroglial cells. Cerebrospinal fluid (CSF) is a reflection of CNS metabolism, and soluble, immunosuppressive factors have been identified in this fluid (Wilbanks 1992). The mitogen-stimulated proliferation of lymphocytes can be influenced in the presence of soluble CSF-derived factors (Taylor 1996).

Equine Protozoal Myeloencephalitis (EPM) is the most frequently diagnosed disease of the central nervous system (CNS) of horses in the United States. EPM is caused by a protozoan organism of the genus Sarcocystis (sp.: S. neurona), and the horse is not the natural intermediate host, rather an aberrant host. It becomes infected by ingestion of infectious sporocysts, which are derived from the sexual replication of the organism in the intestinal tract of the American opossum (Didelphis virginiana). The site of infection in the horse is atypical for Sarcocystis spp. The protozoan organism enters the CNS of horses, and causes lesions presumably during invasion and replication. The presence of intrathecal antibodies as detected by western blot (WB) is used as a diagnostic aid in combination with clinical signs, which most commonly include an asymmetric ataxia.

The current recommended treatment comprises a combination of sulfonamide antibiotics and pyrimethamine for an average duration of six months. Western blot analysis is also used to monitor response to therapy. Absence of measurable intrathecal antibodies is considered representative of resolution of CNS infection. Remission of clinical signs

however, typically precedes negative WB analysis. Further, some clinically normal animals continue to maintain measurable antibody concentrations even after prolonged treatment time (>12 months) (Fenger 1998a). The need for prolonged treatment suggests that the elimination of the Sarcocystis infection by the equine immune system is at best inefficient. There are two possible explanations for this observation. It is possible that the protozoan pathogen, S. neurona, induces an immune suppressive effect, as described for other protozoan organisms (Ming 1995). The second possible explanation focuses on the immune privilege of the CNS. Since T lymphocytes play an important role in the elimination of protozoan organisms (Abbas 1994) this study investigates the potential immune suppressive effect of equine CSF on mitogen-(concanavalin A) stimulated lymphocytes. We also addressed potential differences between the CSF from EPM-affected and control horses. To accomplish this, peripheral blood mononuclear cells (PBMCs) from an EPM-affected and a control horse were collected at different occasions. The *in vitro* response to mitogenic stimulation with ConA was measured when cells were co-cultured with CSF which was fractionated according to the molecular weight of its components.

The results suggest an immunosuppressive, cell donor-dependent effect of CSF on equine PBMCs. The cell donor dependent effect suggests differences in T lymphocyte subsets among the horses that were used as cell donors. These findings suggest a role of a suppressive environment and lymphocyte composition in the delay of pathogen elimination in horses with EPM.

### **Material and Methods:**

#### ***HORSES:***

Sixteen horses of different breeds were used in this study. The horses' ages ranged between 2 and 20 years. Two of the sixteen horses, a 2 year old colt and a 4 year old filly, served as PBMC donors. The colt was the EPM-affected cell donor, while the filly served as the control. Although preference would have been given to same gender, age and breed

regarding the two cell donors, given the stringent criteria for EPM diagnosis it was not possible to find a better match. EPM was diagnosed by clinical signs of the disease coupled with the presence of intrathecal antibodies as detected by Western blot analysis (Equine Biodiagnostics, Lexington KY). All EPM-affected horses (n=11) that served as CSF donors were euthanized after completion of the experimental phase. Gross pathology and histologic examination of the CNS was performed to further support the EPM diagnosis (Mayhew 1978). The clinical signs of the EPM-affected horses varied in their severity. The criteria for selection was based on at least a grade 2/5 ataxia, and conscious-proprioceptive deficits. All of the EPM-affected horses demonstrated weakness during a hind limb sway test. All the horses, except one, which were used as CSF donors of the EPM-affected group were treated with a combination of sulfonamide antibiotics and pyrimethamine for at least 3 months prior to enrollment in this project. All treated horses showed improvement, however, not to a previous level of performance. None of the horses received treatment three months prior to CSF collection. During this period 5 of the 11 horses showed slow, progressive worsening of ataxia, increasing hind limb paresis, and general loss of muscle mass. Control horses were selected based on absence of signs of clinical neurologic disease as determined by two independent clinicians, and on negative immunoblot (WB) results on serum and CSF samples. The gender distribution of the 11 CSF donors affected with EPM was nine geldings and two mares. The three control horses were one gelding and two mares. Horses were maintained on pasture or stall confined with daily turnout. A diet of hay (ad libitum) and water was provided. All maintenance and care were in accordance with the animal welfare guidelines established at the Virginia Polytechnic Institute and State University.

#### *Cerebrospinal Fluid Collection and Processing:*

Approximately 50 ml of CSF was collected at the atlanto-occipital site under general anesthesia (Mayhew 1989) from the EPM-affected and control horses assigned as CSF donors. Three ml of CSF in EDTA were submitted for cytologic evaluation, and a 5 ml

sample without added anti-coagulant was used for protein measurements using a spectrophotometric method that uses a pyrogallol red-molybdate complex as a binding substrate for basic amino acid groups of protein molecules (Microprotein-PR™, Sigma, St. Louis MO). A third CSF sample was submitted for *Sarcocystis neurona* -antibody detection by immunoblot (Western blot) analysis (Equine Biodiagnostics, Lexington, KY; Neogen Inc., Lexington KY). Approximately 35 ml of the remaining CSF were centrifuged at 1500 x g (17 °C, 10 minutes) to remove cells and cellular fragments. The supernatant was frozen at -15 °C in 7 ml aliquots. For each experiment CSF aliquots from a maximum of 4 EPM-affected horses and one control were thawed at room temperature. The fractionation technique was similar to previously described techniques (Taylor 1996). Five ml were transferred into a filtration device with a Biomax low protein binding membrane with a nominal molecular weight limit of 10 kD (Millipore Ultrafree-15, Sigma-Aldrich, St. Louis, MO), and centrifuged for 15 minutes (1500 x g, 17 °C). The filtrate was placed over a second filtration device, this time with a nominal molecular weight limit of 5 kD (Millipore Ultrafree-15 Biomax low protein binding membrane, Sigma-Aldrich, St. Louis, MO), and centrifuged for 15 minutes (1500 x g, 17 °C). Filtering the CSF over two membranes resulted in three fractions of CSF. Fraction-1 contained concentrated substances with a molecular weight higher than 10 kD, fraction-2 contained concentrated substances with a molecular weight between 5 and 10 kD, and fraction-3 contained a filtrate with substances lower than 5 kD. The protein concentrations were measured in the original CSF sample, fraction-1 and fraction-3. With a known protein concentration in the original sample, fraction-1 and 3, and a known volume which was recorded after each filtration step, it was possible to calculate the total amount of protein in fraction-1 and 3. The protein amount in fraction-2 was estimated after subtraction of the sum calculated from fraction-1 and 3 from the total amount of protein in the original sample [F2 = total protein CSF - (F1 + F3)]. This was done to evaluate the correlation between change of proliferative response

and protein concentration. The sum of F1, F2 and F3 should equal the total protein concentration in the original sample.

*Peripheral Blood Mononuclear Cell (PBMC) collection and isolation:*

For each experiment, (i.e. three experiments total) approximately 80 ml of blood was collected from the two PBMC donor horses (i.e. from EPM horse and control). PBMCs were isolated using a modification of previously described methods (Ansar Ahmed 1993). Peripheral blood was collected into heparinized tubes (Vacutainer™, Becton Dickinson, Franklin Lakes, NJ) by aseptic venipuncture from the left or right jugular vein. Routine leukocyte counts (CBC) and leukocyte differentials were performed from separate EDTA- blood samples. The heparinized blood was centrifuged first at 600 x g for 10 minutes. The buffy coat was recovered and re-suspended collectively in 8 ml incomplete RPMI 1640 medium. One ml of the cell suspension (approximately  $20 \times 10^6$  leukocytes) was placed over of 3 ml of ficoll-hypaque (Lymphoprep™, Nycomed Pharma AS, Oslo, Norway) in sterile 15 ml centrifuge tubes at room temperature. Separation of lymphocytes was achieved by continuous gradient centrifugation at 350 x g, 30 minutes at 20°C. Cells were recovered at the ficoll-hypaque cell suspension-interface by gentle aspiration, and re-suspended in incomplete RPMI 1640 medium. The cells were washed three times, and stored in complete RPMI 1640 medium (Cellgro™ RPMI 1640 1x (Mod.) with L-glutamine, Hepes Buffer 25 mM, 10% heat inactivated fetal bovine serum, and penicillin (50 IU/ml)-streptomycin (50 µg/ml)) on ice for no more than one hour. Following cell count with hemacytometer and cytologic evaluation using a Trypan Blue exclusion method (trypan Blue 0.4%, Sigma, St. Louis MO) and a modified Wright-Giemsa staining (Sigma, St.Louis MO), complete RPMI 1640 medium was added to achieve a final concentration of  $5 \times 10^6$  mononucleated cells/ml.

*Lymphocyte Proliferation Assay:*

The proliferation assay was prepared as described previously (Gogal 1997), and the experimental design is in Figure 1. A 1000 µl individual stock solution for each CSF

fraction was prepared. One hundred  $\mu\text{l}$  of each of the CSF fractions and 10  $\mu\text{l}$  of Concanavalin-A (stock concentration: 0.5  $\mu\text{g}/\mu\text{l}$ ) were added to 890  $\mu\text{l}$  RPMI. One hundred  $\mu\text{l}$  of phosphate-buffered saline (PBS) served as a control, used instead of an equivalent volume of CSF. One hundred  $\mu\text{l}$  of the cell suspension ( $5 \times 10^5$  cells) from the EPM horse and the control horse were placed in 96-well round-bottom tissue culture plates (Corning Glass Works, Corning NY), and incubated with 100  $\mu\text{l}$  of the prepared co-culture media, including the mitogen, and incubated at 37°C, 5% CO<sub>2</sub> (Nam 1995, Taylor 1996). After 48 hours of incubation the samples were pulsed with 1  $\mu\text{Ci}/\text{well}$  of <sup>3</sup>H-thymidine, and incubated at 37°C (5% CO<sub>2</sub>) for an additional 18- 20 hours. The cells were harvested with a PHD Cell Harvester (Cambridge Technology Inc., Cambridge MA.) over microfiber filters (Whitman Glass Microfiber Filters), and placed in scintillation vials (Wheaton Scientific, Millville NJ). Each vial was mixed with 3 ml scintillation fluid (Ecoscint™, National Diagnostics, Atlanta GA), and all vials were placed in a Beckman LS 8100 liquid scintillation counter, where beta-emission was counted for ten minutes each vial. Each sample was performed in triplicate. Results were expressed in counts per minute (CPM), and the mean and standard error of mean (SEM) were calculated. CPMs obtained from co-culture of mitogen stimulated PBMCs with saline (PBS) was considered the standard (100%). The results in CPM obtained from co-culture with one of the three CSF fractions were divided by the standard results in CPMs, multiplied by 100, and expressed as a percentage [response in % = CPM(CSF) / CPM(Saline) x 100].

A similar experimental design was used in a preliminary study. Unprocessed CSF from 6 randomly selected horses was used in co-culture with mitogen-stimulated PBMCs from 6 different horses. CSF replaced 5% of the complete RPMI 1640 medium, including mitogen and PBMCs. Different from the experimental design described above the controls lack a correction for media replacement with CSF, using saline.

*Cell Viability Assay:*

To eliminate the possibility that proliferation was limited due to an increase in cell death, cellular viability after 24, 48 and 72 hours of incubation was assessed by routine Trypan Blue exclusion (Trypan Blue 0.4%, Sigma-Aldrich, St. Louis, MO) staining technique. Blood was obtained from 5 randomly selected horses. PBMCs were isolated and cultured with each of the three fractions of one CSF sample and a PBS control. One CSF sample from an EPM-affected horse was selected for this purpose, that previously showed the most profound response during a proliferation assay. The percentage of dead cells was calculated, and compared between groups. Also, PBMCs were co-cultured in the presence of CSF, followed by the measurements of spontaneous proliferation (without an added mitogen) by <sup>3</sup>H-Thymidine incorporation assay. CPMs were compared between PBMCs in complete RPMI 1640 medium, and PBMCs in medium co-cultured with CSF (Table 4).

*Statistical Analysis:*

Statistical analysis included a Wilcoxon signed rank test, followed by the Bonferroni correction multiplying the nominal p-value by the number of observations. This was done to identify differences of a single CSF fraction from the saline control for either the EPM affected or the control horse. A Friedman test was used to compare the results of the corresponding fractions between the two cell donors (EPM vs. control cells). Correlation between protein concentration in CSF and response of PBMCs was calculated using a standard desktop statistical program (Minitab™, College Station, PA). A Student's t-Test was used for the cell viability study between groups. The level of significance for each test was pre-set at p 0.05.

**Results:**

*Lymphocyte Isolation and Recovery:*

The leukocyte counts of the EPM-affected and control blood donors on the three days of blood collection was used as a general indicator of infection / inflammation. For all experiments the cell count and differential were within normal limits ( $7.2 \pm 0.4 \times 10^9$

WBC/L). A mild eosinophilia was noticed in the control horse (up to 5% eosinophils). Her proliferative response of PBMCs to increasing concentrations of ConA appeared not different from other horses. In general recovery of lymphocytes fluctuated between 30 and 40% on different sampling days, however, purity of the separated cells was constant at > 90% mononucleated cells, of which 80 - 90% lymphocytes. The separated fractions also contained a moderate number of platelets.

*CSF analysis and CSF processing:*

CSF analysis of EPM-affected horses and controls were within normal limits. Total CSF protein concentration was between 110 and 690 mg/L (normal: 500-1000 mg/L (Mayhew 1989), and the cell count on CSF was <1 WBC/ $\mu$ l for both groups of horses. The centrifugation combined with filtration of the CSF samples (fractionation) resulted in a significantly unequal distribution of protein amounts among the three fractions. The averaged retained volume of fluid after the filtration processes was for fraction-1: 500  $\mu$ l; fraction-2: 300 $\mu$ l; fraction-3: 3 ml. Calculation of the total amount of protein within the initial CSF volume of 5 ml, and total amount of protein in the remaining sample after the first filtration step was similar (original CSF sample (2.03 mg  $\pm$  0.2) and fraction 1 (1.95 mg  $\pm$  0.19)). Most of the protein was therefore retained in fraction-1. Only a minuscule amount (20  $\mu$ g  $\pm$  2) could be detected in fraction-3. Using the formula as described in Material and Methods, the total amount of protein in F2 was approximated at 60  $\mu$ g.

*Lymphocyte Proliferation Assay:*

Mitogen-stimulated PBMCs from the EPM-affected horse and the control showed significant differences in proliferation when CSF fractions were added. Figures 2a and 2b show the results of the proliferation assay, where the proliferative response of PBMCs was compared between CSF fractions (F1, F2, F3) and the saline control. All three fractions, derived from CSF of EPM-affected horses, showed a significant ( $p < 0.05$ ) decrease when co-cultured with PBMCs from the EPM horse (Figure 2a). However, when the identical CSF fractions were added to PBMCs from the control horse the response was different

(Figure 2b). A difference in proliferative response between the two cell donors was most noticeable in F1. PBMCs from the EPM-affected horse showed a significant decrease in their response to the mitogen ( $p < 0.05$ ), while PBMCs from the control horse actually showed increased proliferation ( $p < 0.05$ ). PBMCs from the control horse co-cultured with fraction 2 and 3 resulted in an insignificant decrease in proliferation due to a greater variation among the individual results. Mitogen-stimulated PBMCs from the EPM-affected and control horse co-cultured with the CSF-fractions from control horses showed similar cell-donor dependent results, as observed for the CSF fractions from EPM-affected horses (data not shown).

Results from the preliminary study, where unprocessed CSF was used, are presented in Table 3. In two out of six randomly selected horses a significant decrease in mitogen-stimulated lymphocyte proliferation was seen when co-cultured with unprocessed equine CSF.

*Viability Assessment:*

The viability staining with Trypan Blue of PBMCs in complete RPMI 1640 medium, and in co-culture with CSF fractions F1, F2 and F3 did not show an increase in dead cells (Figure 3). The results of the cells cultured in absence of a mitogen stimulant, but in co-culture with CSF were compared to cells in medium only. Differences in spontaneous proliferation as measured by a  $^3\text{H}$ -Thymidine assay, were not noticed ( $p > 0.05$ ) (Table 4).

*Post-mortem evaluation including histopathology :*

At least one of the histopathologic samples from each horse submitted for microscopic evaluation revealed mild to moderate changes in spinal cord segments suggestive of an infection with *S. neurona*. The findings included mild to moderate gliosis, occasional perivascular cuffing, and neuronal vacuolization. An asymmetric distribution of the findings was noticed, and the majority of lesions were found in the ventral white matter of the submitted spinal cord segment. One horse had a hemorrhagic area in a cortical

segment, in addition to findings in one spinal cord segment. Protozoan stages were not detected in any of the histologic sections.

### **Discussion:**

Currently, there is limited information about the interaction between the protozoan organism and the horse. This study is the first in its kind focusing on the microenvironment at the infection site in horses with EPM. Some results of this study suggest a role of the central nervous microenvironment in the course of the disease which potentially could explain the prolonged treatment interval, and slow recovery of horses with EPM. The decreased proliferative response of mitogen-stimulated lymphocytes indicates the presence of soluble factors with an immune suppressive effect in the CNS of horses. Because mononuclear cells play an important role in the pathogen-host defense mechanism in EPM, the presence of these factors in CSF may therefore not only influence an *in vitro* response. Decreased proliferative response of lymphocytes when co-cultured with CSF had been demonstrated in previous studies (Wilbanks 1992, Taylor 1996). Soluble factors with the potential to influence lymphocyte proliferation were identified as Transforming Growth Factor- $\beta$  (TGF- $\beta$ ), Interleukin-10,  $\alpha$ -Melanocyte Stimulation Hormone ( $\alpha$ -MSH), and Vasoactive Intestinal Peptide (VIP). Taylor identified TGF- $\beta$  as a factor with potential immunosuppressive activity in CSF. Blocking TGF- $\beta$  action by anti-TGF antibodies showed a reversion to comparable lymphocyte proliferation, as demonstrated in assays where CSF was not added. TGF- $\beta$  is a potent immunoregulator, and its main function is suppressing the clonal expansion of T helper 1 cells, and promoting a T helper 2 response. TGF- $\beta$  is continuously expressed at immune privileged sites, which includes the CNS (Flaumenhaft 1993), however, predominantly in its latent form. TGF- $\beta$  has to become activated first by proteases for its immunosuppressive effect, and the *in vivo* trigger mechanism for this activation is yet unknown (Letterio 1997). The ratio between active and latent TGF- $\beta$  can increase during disease or trauma (Keane 1997). Besides an interference

during antigen presentation and clonal expansion, TGF- $\beta$  also suppresses the expression of adhesion molecules (ICAM, VCAM) and MHC II on astrocytes. It decreases the proliferation of astrocytes, and stimulates oligodendrocyte and Schwann cells to proliferate, which are important cells for myelin production during neuronal repair (Otero 1994). A very important cytokine from T helper 1 cells is Interferon- $\gamma$ . Its role in host defense regarding intracellular pathogens is well documented. A number of publications identify the importance of Interferon- $\gamma$  in protozoan infections, and specifically in toxoplasmic encephalitis. By down-regulating a T helper 1 cell response the most potent cytokine associated with killing of intracellular pathogens disappears in presence of high concentrations of TGF- $\beta$  (Hunter 1994, Hunter 1996, Alexander 1997, Daubner 1997). Other factors with an immune suppressive effect that were found in the CNS include  $\alpha$ -MSH (Catania 1993), Vasoactive Intestinal Peptide (VIP) (Catania 1993), and Interleukin-10 (IL-10) (Frei 1994). Their presence in humans and laboratory animals is documented, and they contribute to the immune suppressive effect at immune privileged sites. Equine specific IL-10 or TGF- $\beta$  has not yet been identified, however, the T helper cell dichotomy based on cytokine profiles has been established in horses (Swiderski 1998).

In our study protein molecules lower than 10 kD demonstrated the most profound effect on lymphocytes in culture. Substances such as  $\alpha$ -MSH and VIP have a molecular weight lower than 10 kD, which makes them possible candidates for further investigation. Both molecules were detected in the equine;  $\alpha$ -MSH was also found in equine CSF (Wilson 1982). Taylor was able to show that minuscule amounts of these substances were able to cause decrease in lymphocyte proliferation. This supports our findings that the most profound decrease in lymphocyte proliferation in co-culture was noticed in fraction 2 and 3, and also explains why the fractions with a very low measurable protein concentration still showed suppression of proliferation (Taylor 1996). Catania *et al.* found an anti  $\alpha$ -MSH molecule in human CSF (Catania 1993). The anti  $\alpha$ -MSH had a molecular weight between 20 - 40 kD, and was able to bind free  $\alpha$ -MSH. With the artificial separation of CSF

proteins in our study some of the decrease in proliferation could be attributed to such an effect. However, unprocessed CSF showed a decreased proliferation in 2 out of 6 horses; and it would not explain the cell donor dependent differences that were noticed.

In our study possible TGF- or IL10 equivalents, based on their molecular weight should be found in fraction 1, where less of a response was noticed. Latent and activated human TGF- are larger than 10 kD, therefore with the assumption of an equivalent molecule in the horse, the molecules would be found in fraction-1. T helper cell dichotomy is a potential explanation for the findings in this study. A factor such as TGF- that influences only a particular group of T cells can cause an overall decrease in proliferation of mitogen-stimulated lymphocytes depending on the ratio between responsive and non-responsive T cells. Hypothesizing that not each horse has the same T helper cell subsets at any moment in time, our results may suggest the presence of a selectively suppressing factor in CSF, that interacts with a particular group of T cells. These differences could depend on gender, breed, or age. However, different T H subtypes could also be induced by diseases such as EPM. Besides further CSF analysis identifying specific immunosuppressive factors, T cell subset should be analyzed in reference to whether a horse has EPM or not.

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**Table 1:** Protein amounts in CSF and processed CSF fractions, listed for each individual horse. Protein in mg and calculated for the available volume of fluid: original CSF (column 2), fractions 1 (column 3), fraction 3 (column 4) for EPM affected and control horses (n = 14). Notice that results for fraction 2 can be estimated: [F2= CSF-protein -(F1 + F3)].

Horse	Amount of CSF protein in mg per 5 ml: original CSF	Amount of CSF protein in mg in 0.5 ml: F1	Amount of CSF protein in mg in 3ml: F3
EPM 1	1.35	2.14	0.02
EPM 2	0.55	2.11	0.019
EPM 3	1.95	2.3	0.021
EPM 4	2.75	1.57	0.028
EPM 5	1.65	2.6	0.019
EPM 6	1.55	1.1	0.026
EPM 7	1.35	0.64	0.012
EPM 8	2.05	1.5	0.019
EPM 9	2.35	3.7	0.018
EPM 10	2.70	1.87	0.029
EPM 11	2.45	2.0	0.022
Control 1	3.45	2.2	0.015
Control 2	1.70	1.4	0.02
Control 3	2.50	2.1	0.005
n= 14 (Mean ± SEM)	2.03 ± 0.2	1.95 ± 0.19	0.02 ± 0.002

**Table 2a:** Proliferation of PBMCs co-cultured with CSF fractions (columns 2, 3, 4) compared to PBMCs co-cultured with equal volume of saline. Source of PBMCs: EPM affected horse. Source of CSF: EPM-affected (n=11), and control horses (n=3). Results expressed as a percentage.

Horse	Proliferation in percent compared to saline control (100%) F1	Proliferation in percent compared to saline control (100%) F2	Proliferation in percent compared to saline control (100%) F3
EPM 1	81	71	67
EPM 2	97	77	79
EPM 3	78	87	78
EPM 4	70	46	56
EPM 5	71	69	55
EPM 6	58	56	64
EPM 7	66	56	58
EPM 8	99	66	60
EPM 9	91	68	71
EPM 10	60	52	51
EPM 11	83	59	66
n=11 (Mean ± SEM)	77.6 ± 4.3	64.3 ± 3.6	64.1 ± 2.8
Control 1	87	56	63
Control 2	108	80	82
Control 3	91	89	77
n= 3 (Mean ± SEM)	95 ± 6.4	75 ± 9.9	74 ± 5.7

**Table 2b:** Proliferation of PBMCs co-cultured with CSF fractions (columns 2, 3, 4) compared to PBMCs co-cultured with equal volum of saline. Source of PBMCs: Control Horse. Source of CSF: EPM-affected (n=11), and control horses (n=3). Results expressed as a percentage.

PBMCs: control	Proliferation in percent compared to saline control (100%)	Proliferation in percent compared to saline control (100%)	Proliferation in percent compared to saline control (100%)
CSF from:	F1	F2	F3
EPM 1	95	82	68
EPM 2	124	89	90
EPM 3	118	107	104
EPM 4	122	114	121
EPM 5	120	88	67
EPM 6	112	81	72
EPM 7	108	64	47
EPM 8	123	84	78
EPM 9	120	116	93
EPM 10	116	90	74
EPM 11	101	73	60
	114.5 ± 2.9	89.8 ± 5	79.5 ± 6.4
Control 1	105	59	88
Control 2	116	93	85
Control 3	109	86	85
n= 14 (Mean ± SEM)	110 ± 3.2	79.3 ± 10.4	86 ± 1

**Table 3:** Co-culture of mitogen-stimulated PBMCs with complete CSF. Results expressed as counts per minute (CPM) (column 2 and 3), or as a percentage [(column 3 / column 2) x 100]. Column 2 served as a control (100%).

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Pilot Study:

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Results in CPM	mitogen-stimulated PBMCs in RPMI 1640	mitogen-stimulated PBMCs in RPMI 1640, co-cultured with CSF	proliferation in percent compared to medium control
Horse 1	35680	25180	71
Horse 2	79480	74440	94
Horse 3	34600	31610	91
Horse 4	37220	38540	104
Horse 5	66420	65235	98
Horse 6	10870	7710	71

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**Table 4:** Spontaneous proliferation of PBMCs from n=6 horses cultured in complete RPMI 1640 medium alone, or in co-culture with medium and unprocessed CSF. Differences in percent are expressed in column 3.

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	CPM of PBMCs in cRPMI 1640, no mitogen	CPM of PBMCs in cRPMI 1640 and CSF, no mitogen	Differences in percent: [(CPM CSF/ CPM Media) x 100]
Horse 1	2740	2880	105
Horse 2	1800	2600	144
Horse 3	4640	6705	144
Horse 4	3970	3420	86
Horse 5	2125	1898	89
Horse 6	1355	1163	86

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## 7. General Summary and Conclusions:

The main body of research in Equine Protozoal Myeloencephalitis over the past 30 years has focused on studies in epidemiology, drug efficacy studies, and determining the life cycle of *Sarcocystis neurona*. Mechanisms of pathogen-host interactions are still under investigation, and are therefore poorly understood. The interaction of the Equine immune system with the parasite, and the nature of the CNS microenvironment were the primary foci of the study reported herein.

In general, protozoan organisms are frequently observed as opportunistic infections, and interestingly, the central nervous system (CNS) is the only organ system in the horse where *S. neurona* has been found. Further, the immune response of the CNS in humans and other mammals is ameliorated when compared to other body systems. This decreased responsiveness is maintained by the presence of immunosuppressive factors, and an impediment of cellular access to the CNS. It were these two observations that lead us to investigate whether there is a pathogen-associated mechanism which influences the efficacy of the immune response, and whether the microenvironment at the site of infection influences the course of the disease.

The results from both studies (Experiment 1 and 2) suggest that the pathogen and the site of infection have an effect on the pathogenesis of EPM. Both experiments showed results with important preliminary findings for further research. T lymphocytes play an important role in host defense mechanisms, and in both studies we demonstrated a significant effect on these cells. A decrease in proliferation of lymphocytes in co-culture with fragment of *S. neurona*, and a cell donor dependent effect on mitogen-stimulated lymphocytes in co-culture with cerebrospinal fluid were noticed.

We proposed a mechanism where the protozoan organism induces the production of a specific factor, which decreases the efficiency of the host's immune system. Also, based on the results of the second experiment, specific immunosuppressive factors might be

already present at the site of infection, which further facilitates the pathogen's presence and reproductive ability at that site. We hypothesize that there is a complex and decreased immune response efficacy due to location of the infection, and because of a specific mechanism of the pathogen (infectious quality) that is impeding efficacy of therapy. Shortening of the disease process may be achieved by counteracting the mechanisms of immune impairment which were detected in these two studies. Effective therapeutics have to be combined with molecular engineered products that are able to steer and counteract immune processes in pathogen-host interaction

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Equine Protozoal Myeloencephalitis. Preliminary Investigation of Protozoan-Host  
Interaction in the Horse

Doctorandus (drs.) in Veterinary Medicine, September 1993  
University of Utrecht, The Netherlands

Abitur, Salier Gymnasium, May 1982  
Waiblingen, Germany

### Specialty Board Certification:

Board Eligible: American College of Veterinary Internal Medicine

Employment: Virginia-Maryland Regional College of Veterinary Medicine,  
Marion duPont Scott Equine Medical Center  
Leesburg, Virginia  
August 1995 - July 1998

Dr. M. Englisch, Equine Veterinary Services, Nürnberg, Germany  
June 1993 - July 1995

### Professional Organization Membership:

Royal Dutch Veterinary Medical Association 1987 - present  
International Society of Veterinary Perinatology 1990 - present

### Related Experience:

Equine Protozoal Myeloencephalitis. Preliminary Investigation of Protozoan-Host  
Interaction in the Horse.

Seminar: James A. Baker Institute for Animal Health, College of Veterinary  
Medicine, Cornell University, New York, 1998

Effect of Cerebrospinal Fluid from Horses with Equine Protozoal  
Myeloencephalitis on Equine Lymphocyte Proliferation.  
Presentation at 8th International Conference in Equine Infectious Diseases,  
Dubai (UEA) 1998

Sarcocystis neurona and Equine Protozoal Myeloencephalitis  
Seminar: College of Veterinary Medicine, Zurich University, Switzerland,  
September 1997

