Investigation of Immune Response to *Sarcocystis neurona* Infection in Horses with Equine Protozoal Myeloencephalitis

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Abstract

Equine Protozoal Myeloencephalitis (EPM) is a serious neurologic disease of horses in the United States. The primary etiologic agent is *Sarcocystis neurona* (*S. neurona*). Currently, there is limited knowledge regarding the protective or pathologic immune response to infection to the intracellular protozoa *S. neurona*. The objective of these studies was to determine the effects of *S. neurona* infection on the immune response of horses that had EPM due to natural infection (experiment 1) and experimental infection (experiment 2). In experiment 1, twenty-two horses with naturally occurring cases of EPM, which were confirmed positive based on detection of antibodies in the serum and/or CSF and clinical signs, and 20 clinically normal horses were included to determine whether *S. neurona* altered the immune responses, as measured by immune cell subsets (CD4, CD8, B-cell, monocytes, and neutrophils) and leukocyte proliferation (antigen specific and non-specific mitogens). Our results demonstrated that naturally infected horses had significantly higher percentages of CD4 and neutrophils (PMN) in peripheral blood mononuclear cells (PBMCs) than clinically normal horses. Leukocytes from naturally infected EPM horses had a significantly lower proliferation response, as measured by thymidine incorporation, to a non-antigen specific mitogen phorbol 12-myristate 13-acetate (PMA) / ionomycin (I) than did clinically normal horses ($p=0.04$). The implications of these findings will be discussed.

In experiment 2, 13 horses were randomly divided into two groups. Baseline neurologic examinations were performed and all horses were confirmed negative for *S. neurona* antibodies in the CSF and serum. Then, one group with 8 clinically normal seronegative horses was inoculated intravenously with approximately 6000 *S. neurona* infected autologous leukocytes daily for 14 days. All the challenged horses showed neurologic signs consistent with EPM. PBMCs were isolated from the control and infected horses to determine how *S. neurona* alters the immune responses based on changes in immune cell subsets and immune function. There were no significant differences in the percentage of CD4 cells in peripheral blood lymphocytes or IFN-γ production by CD4 and/or CD8 cells. PMA/I stimulated proliferation responses in PBMCs appeared suppressed compared to that of uninfected controls. Additional studies are necessary to determine the role of CD4 and CD8 cells in disease and protection to *S. neurona* in horses, as well as to determine the mechanism associated with suppressed *in vitro* proliferation responses. This project was funded by Patricia Stuart Equine grants and paramutual racing funds from Virginia Tech.

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TABLE OF CONTENTS

TITLE PAGE ……i

ABSTRACT ……...ii

ACKNOWLEDGEMENTS ……iv

TABLE OF CONTENTS ……v

LIST OF FIGURES ……vii

LIST OF TABLES ……...ix

LIST OF ABBREVIATIONS ……..x

CHAPTER 1 Introduction ……..1

CHAPTER 2 Literature review of Equine Protozoal Myeloencephalitis …...3

2.1 History and etiologic agent ……3
2.2 Life cycle of *Sarcocystis neurona* ……..5
2.3 Epidemiology ……8
2.4 Experimental Animal Models……11
2.5 Clinical signs……18
2.6 Diagnosis……19
2.6.1 Cytology and Cerebrospinal fluid indices (CSF/indices)……20
2.6.2 Enzyme-linked immunosorbent assay (ELISA) to *Sarcocystis neurona* Surface Antigen 1 (SnSAG1)……..23
2.6.3 Immunoblot assay (western blot)……25
2.6.4 Immunofluorescence Assay (IFA)……26
2.6.5 Serum Agglutination Test (SAT)……26
2.6.6 Polymerase Chain Reaction (PCR)……27
2.7 Pathology……..27
2.8 Treatment……..28
2.9 Prevention……30
2.10 Future goals……31

CHAPTER 3 Immunophenotypes and lymphocyte proliferative responses in naturally infected EPM horses ……..34

3.1 Introduction ……34
3.2 Materials and methods ……35
3.2.1 Animals ……35
3.2.2 Collection of blood samples……36

3.2.3 Differential cell counts ……..36
3.2.4 Isolation of Peripheral Blood Mononuclear Cells (PBMCs)..……...…37
3.2.5 Cytospin preparation ……..…….37
3.2.6 Flow cytometry ……..38
3.2.7 7-amino actinomycin D (7-AAD) staining…….39
3.2.8 Live merozoite preparation ……..……...…39
3.2.9 Lymphocyte Proliferation Assays……...40
3.2.10 SnSAG-1 (*Sarcocystis neurona* surface antigen-1) plate incubation …..41
3.3 Statistical Analysis ……..……41
3.4 Results ……..42
3.4.1 Effects of storage or shipping condition on immunological assays utilizing equine PBMCs……...42
3.4.2 Analysis of immune cell subsets and changes in immune function between naturally infected EPM and clinically normal horses……44
3.5 Discussion ……...……45

CHAPTER 4 Changes in immune subsets and function associated with experimental *S. neurona* infection of horses ……62

4.1 Introduction ……62
4.2 Materials and Methods ……64
4.2.1 Animals ……..……64
4.2.2 Isolation of Peripheral Blood Mononuclear Cells (PBMCs)..……...66
4.2.3 Cytospin preparation ……..….66
4.2.4 Flow cytometry ……..……67
4.2.5 7-AAD staining ……..67
4.2.6 Live merozoite preparation …….....……68
4.2.7 Lymphocyte proliferation assays ……...68
4.2.8 SnSAG-1 plate incubation …….....69
4.2.9 γ-IFN intracellular cytokine staining ……..……69
4.2.10 PMA/I induced lymphocyte apoptosis……..…….....…70
4.3 Statistical Analysis..……..……71
4.4 Results..……71
4.4.1 Effects of *S. neurona* infection on immunophenotypes of experimentally infected horses ……..71
4.4.2 Analysis of lymphocyte proliferation assays ……....73
4.4.3 Analysis of γ-IFN intracellular cytokine staining ……..……...73
4.4.4 Effects of PMA/I on lymphocyte apoptosis ……..…....74
4.5 Discussion..……...…..74

CHAPTER 5 General Summary and Conclusions ……....101

FUTURE STUDIES ……..…105

LITERATURE CITED ……108

LIST OF FIGURES

Figure 2.1 Phylogenetic classification of *Sarcocystis spp* ……....4

Figure 2.2 Life cycle of *Sarcocystis neurona* ……...7

Figure 2.3 CSF-indices ……...22

Figure 2.4 Calculation of AI SAG1 index ……..…24

Figure 3.1 Percentage of CD8 T cells of total lymphocytes ……..54

Figure 3.2 Pokeweed (PWM) stimulated proliferation assay comparing normal and EPM affected horses ……..…56

Figure 3.3 Blastogenesis assays stimulated by live *S. neurona* merozoites..…...…57

Figure 3.4 Percentage of CD4 cells of total lymphocytes between normal and EPM affected horses ……58

Figure 3.5 Percentage of neutrophils of total leukocytes ……59

Figure 3.6 PMA/I stimulated proliferation assay comparing normal and EPM horses ……..…60

Figure 3.7 *S. neurona* merozoite stimulated proliferation assay comparing normal and EPM horses……....61

Figure 4.1 Changes in the percentages of CD4 lymphocytes between *S. neurona* challenged horses and non-challenged horses ….....84

Figure 4.2 Changes in the percentages of CD8 lymphocytes between *S. neurona* challenged and non-challenged horses ……..85

Figure 4.3 Changes in the percentages of B-cell lymphocytes between *S. neurona* challenged horses and non-challenged horses ……86

Figure 4.4 Proliferation data from cells stimulated by PMA/Ionomycin in experimentally infected vs control horses…….…….88

Figure 4.5a The percentage of γ-IFN positive CD4 cells as a percentage of the total CD4 T cells incubated with ConA……...…90

Figure 4.5b The percentage of γ-IFN positive purified CD4 cells of the total lymphocytes incubated with ConA ……...……...…91

Figure 4.6 Percentage of CD8 cells in early apoptosis of the total CD8 cells when incubated with PMA/I……...…94

Figure 4.7a Percentage of CD8 cells following overnight stimulation with ConA ($p<0.05$)……95

Figure 4.7b Percentage of CD8 cells following overnight stimulation with merozoites ($p=0.084$)……..96

Figure 4.7c Percentage of CD8 producing γ-IFN following overnight stimulation with ConA ($p=0.10$)……97

Figure 4.7d Percentage of CD8 producing γ-IFN cells following overnight stimulation with merozoites ($p=0.06$)…….98

Figure 4.8a Proliferation data from cells stimulated by *S. neurona* merozoites in experimentally infected vs control horses……....99

Figure 4.8b Proliferation data from cells stimulated by SAG-1 in experimentally infected vs control horses…….....100

LIST OF TABLES

Table 3.1 P-value data for immune cell subsets comparing day 0 and day 1 samples ……..…..….53

Table 3.2 Statistical evaluation of proliferation results (p-value)..…….……..…55

Table 4.1 Results of p-values from blastogenesis assays of experimentally infected horses ……87

Table 4.2 P-value of cytokine data ……..…..89

Table 4.3 Selected p-value data for PMA/Ionomycin apoptosis assay.……..…...92

LIST OF ABBREVIATIONS

7-AAD	7-amino actinomycin D
AQ	albumin quotient
BBB	blood brain barrier
BrdU	5-bromo-2-deoxy-uridine
CNS	central nervous system
ConA	concanavalin A
CPM	counts per minute
CSF	cerebrospinal fluid
CyA	cyclosporin A
DNA	deoxyribonucleic acid
EPM	Equine Protozoal Myeloencephalitis
ERK	extracellular signal-regulated kinase
FBS	fetal bovine serum
FDA	food and drug administration
FITC	fluorescein isothiocyanate
I	ionomycin
IC	immunocompetent
IFA	immunofluorescence assay
γ-IFN	gamma interferon
Ig	immunoglobulin
IFA	immune fluorescence assay
IL	interleukin
KO	knock out
LPS	lipopolysaccharide
NF-κB	nuclear factor-κB
NFAT	nuclear factor of activated T cells
PBMCs	peripheral blood mononuclear cells
PBS	phosphate buffered-saline solution
PCR	polymerase chain reaction
PE	phycoerythrin
PFOR	pyruvate ferredoxin oxidoreductase
PHA	phytohemagglutinin
PI	post infection
PMA	phorbol 12-myristate 13-acetate
PWM	pokeweed mitogen
RBC	red blood cell
RNA	ribonucleic acid
s.c.	subcutaneous
SCID	severe combined immune deficiency
Th	T helper cell
WB	Western Blot

CHAPTER 1 Introduction

Equine Protozoal Myeloencephalitis (EPM), due to *Sarcocystis neurona* infection, is a serious neurologic disease of horses in the United States (US) (NAHMS, 1998; MacKay et al., 2000; Dubey et al., 2001a). Horses, which are aberrant intermediate hosts, are infected through ingestion of contaminated feed stuffs. Clinical signs of horses with EPM typically include ataxia, asymmetric signs, and muscle atrophy (Fenger et al., 1997a; Fenger, 1997b). Currently, a reliable diagnosis of EPM is based on abnormal neurologic signs consistent with EPM and detection of *S. neurona* IgG antibodies in the serum and CSF utilizing the western blot (WB). Treatment for EPM may include one or more anti-protozoal drugs as well as supportive care. Although 70% of horses improve with treatment, which may be needed for months to years, a considerable number of horses do not recover completely. Other horses do not respond or even deteriorate and must be euthanized.

The losses due to EPM are overwhelming. Costs are most commonly due to veterinary services, diagnostics, treatment, time off from performing (training, breeding), loss of winnings, and economic value of the horse, if euthanatized. Estimated costs to diagnose and/or treat a horse with EPM are $400 to $2,400/month depending on treatment choices and other factors (MacKay, 1993; Dubey et al., 2001a). Annual losses in the US are $55.4 to $110.8 million (Dubey et al., 2001a).

If an efficacious vaccine were available, the benefit to the equine industry would be tremendous. At this time, there is only one FDA (U.S. Food and Drug

Administration) conditionally approved vaccine available, which is produced by Fort Dodge Animal Health (FDAH). Data on its efficacy is still pending as results from challenge studies have not been presented or published. In order to develop an efficacious vaccine, it would be ideal to know the protective immune response to *S. neurona* infection. However, at this time, little is known about the protective immune response to *S. neurona* infection in horses. This limits the ability of researchers to understand the pathophysiology of the disease, generate an efficacious vaccine, and develop more sensitive diagnostic assays. Therefore, the overall goal of these studies focused on identifying the changes in immune response due to naturally occurring and experimentally induced cases of EPM as a means of elucidating the protective and/or pathophysiologic immune response associated with EPM. Specific aims include: 1) to determine how *S. neurona* in naturally occurring cases of EPM alters host immune responses based on changes in immune cell subsets and immune function, and 2) to determine how *S. neurona* in experimentally infected horses alters the immune responses based on changes in immune cell subsets and immune function.

CHAPTER 2 Equine Protozoal Myeloencephalitis (EPM) literature review

2.1 History and etiologic agent

The disease "Equine protozoal myeloencephalitis" (EPM) was first named by Mayhew in 1978 (Mayhew et al., 1978). Previously Rooney had seen horses with similar lesions and described it as "*segmental myelitis*" (Rooney et al., 1970). Originally, researchers thought that the disease was caused by a protozoal agent, specifically a *Toxoplasma*-like organism (Beech, 1974; Cusick et al., 1974; Dubey, 1974). However, based on morphologic, ultrastructural, and molecular studies, the organism was demonstrated to be a *Sarcosystis* species, not *Toxoplasma spp* (Simpson and Mayhew, 1980; Dubey et al., 1991; Fenger et al., 1994). In 1991, Dubey was the first person to identify the agent as *Sarcocystis neurona* (*S. neurona*) (Dubey et al., 1991) after isolating it from an infected horse in New York. More recently, *Neospora hughesi* (*N. hughesi*) has also been identified as a causative agent of EPM, but it is much less common than *S. neurona* (Mackay et al., 2000). Once the primary causative agent was identified, "the phylogenetic relationship (Fig. 2.1) of *S. neurona* to other members of the family *Sarcocystidae* was elucidated based on small subunit ribosomal RNA (ssRNA) gene sequence comparisons of cell culture-derived merozoites and sporocysts separated from the intestines of the definite host, the opossum (*Didelphis virginiana*)" (Fenger et al., 1995, 1997; Dubey, 1991; Goehring, 1998).

Phylum:	Apicomplexa
Class:	Sporozoasida
Subclass:	Coccidiasina
Order:	Eucoccidiorida
Suborder:	Eimeriorina
Family:	Sarcocystidae
Subfamily:	Sarcocystinae
Genus:	*Sarcocystis*

Fig 2.1: Classification of *Sarcocystis spp* (Gardiner et al., 1988).

2.2 Life cycle of *Sarcocystis neurona*

The complete life cycle of *S. neurona* in the horse is known, including its developmental cycle and localization within the intermediate host (Dubey et al., 2001a; Fig 2.2). Members of *Sarcocystis spp.* typically have a two host, heteroxenous life cycle. The two hosts usually have an obligatory predator-prey relationship, which means that the definitive host eats the dead body or tissue, which contains *S. neurona*, from the intermediate host. The opossum is the definitive host, and horses are considered aberrant intermediate hosts (Fenger et al., 1997a). The raccoon (*Procyon lotor*) (Stanek et al., 2002), sea otter, armadillo, cat, Fisher, and striped skunk (Cheadle et al., 2001) are all natural intermediate hosts of *S. neurona*. Opossums are the definitive hosts to at least 3 named Sarcocystis species (*S. neurona*, *S. falcatula*, and *S. speeri*) (Box et al., 1984; Dubey, 2000; Dubey et al., 2000a; Dubey et al., 2000b; Dubey et al., 2000c; Dubey et al., 2001a).

S. neurona has a sexual cycle occurring in the intestinal tract of the opossum and asexual reproduction (merogony) with systemic infection in the intermediate host (Dubey et al., 2001a). Ingestion by the definitive host of intramuscular cysts (with bradyzoites) from an intermediate host results in digestion of the sarcocyst wall, invasion of the small intestinal epithelium, and sexual development by gametogony. The newly formed oocysts contain two sporocysts which are passed in the feces and are immediately infectious to the intermediate host. Horses are often infected through ingestion of contaminated feed or water sources. The ingested sporocysts excyst and sporozoites invade the small intestinal endothelium and reproduce

asexually (merogony) to produce schizonts and merozoites. Merozoites migrate or are transported to the CNS, where they continue to undergo merogony intracellularly in neurons and microglial cells (Simpson and Mayhew, 1980; Dubey and Miller, 1986; Bowman et al., 1992). The mechanism by which merozites enter the blood brain barrier (BBB) is not known, but may involve leukocytes and/or endothelial cells (Granstrom and Saville, 1998). After asexual reproduction, the organism develops into sarcocysts in skeletal muscle. Sarcocysts of *S. neurona* have not been found in the horses to date, which indicates that the horse is a dead-end host (Mackay, 2000).

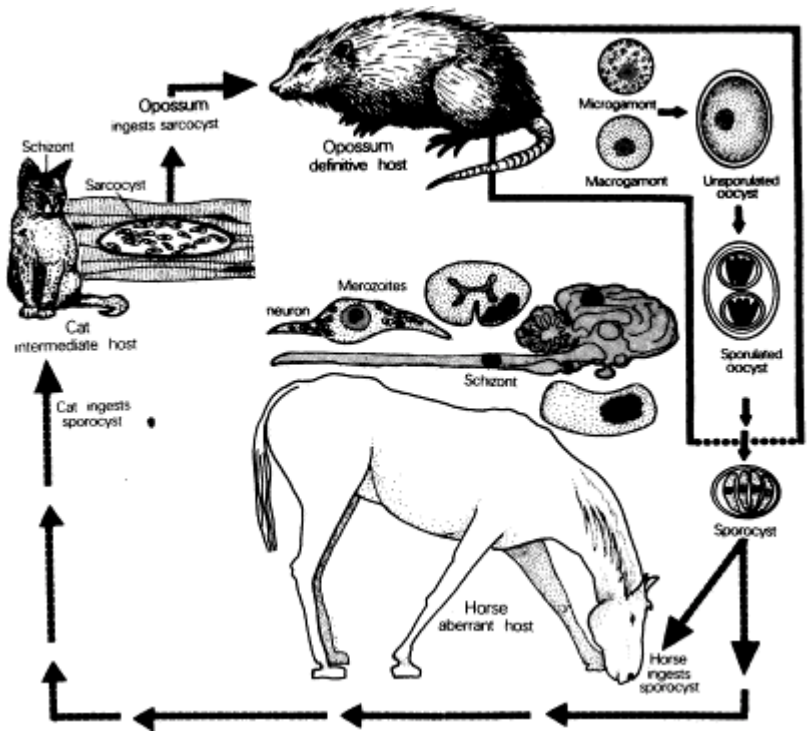

Fig 2.2: Life cycle of *Sarcocystis neurona* demonstrating that the opossum is the definite host and the horse is the aberrant intermediate host (Dubey et al., 2001a, permission obtained from Dr. Dubey).

2.3 Epidemiology

"Knowledge about the epidemiology of EPM advances slowly" (Dubey et al., 2001a). Efforts have focused on identifying the prevalence of disease throughout the world as well as identifying affected populations and associated risk factors. "Cases of EPM have been described predominantly in North, Central and South America" (Goehring, 1998). Early studies have demonstrated that the definitive host (opossum) must be present in order to have cases of EPM. There have been sporadic cases of EPM reported in Europe, South Africa, and Asia, but those cases involved horses that had been exported from the US (Mayhew and Greiner, 1986; Ronen, 1992; Lam et al., 1999; Dubey et al., 1999a; Dubey et al., 1999b; Katayama et al., 2003). This suggests that they must have been infected while in the US.

Recent review articles have shown that about 50% of horses in the US have been exposed to *S. neurona* based on WB tests (MacKay, 1997b; Dubey et al., 2001a). In several different studies, seroprevalence was evaluated in California, Colorado, Oregon, Ohio, and Pennsylvania (Bentz et al., 1997; Blythe et al., 1997; Saville et al., 1997; Tillotson et al., 1999; Vardeleon et al., 2001). Exposure to *S. neurona* among horses in the eastern states appeared to be 10-15% higher than the rest of the states due to the higher prevalence of opossum in the eastern states (Granstrom, 1995).

Besides the presence of *S. neurona* and the definitive host, other factors that influenced prevalence appeared to be climate, breed, and age. With respect to

climate, studies in Oregon and Ohio indicated that coastal areas and places where the temperature was above freezing have higher seroconversion to *S. neurona.* In Oregon, the overall seroprevalence in Oregon was 45%. However, the coastal area was 65% vs 22% in the arid region (Blythe et al., 1997). In Ohio, seroprevalence in southern Ohio was higher than in northern Ohio, which could correlate to the higher number of days above freezing in southern Ohio (Saville et al., 1997). Results of these studies indicated that seroprevalence of antibodies to *S. neurona* in horses increased in wet and warm locations.

Breed may be another factor which influenced infection with *S. neurona*. The first national epidemiologic survey about EPM, conducted by Fayer (Fayer et al., 1990), included 364 histologically confirmed cases. Within their study, the most affected breeds included Thoroughbred, Standardbreds, and Quarter Horses (in that order). By contrast, another retrospective study including 82 EPM cases reported that the risk was higher in Standardbred horses (Boy et al., 1990). Although it was not clear as to why breed differences existed between these studies, one possible explanation may involve geographical differences in breeds present. Overall, those studies suggested that the above mentioned breeds were probably the most commonly affected breeds. However, these studies did not take into account factors such as stress, work load, and/or performance demands.

With respect to age and gender, seroprevalence increased in horses up to 4 years of age and greater than 10 years of age (Mackay, 2000; Saville et al., 2000a, 2000b). This could reflect that chances of exposure increased with time. Additionally,

performance horses are generally younger and people would be likely to detect subtle differences in behavior/performance that may correlate with EPM. Therefore, horses up to 4 years of age may be more likely to be examined by a veterinarian. Horses older than 10, may begin to be more at risk for disease as they could be more easily stressed and therefore susceptible to disease. In one retrospective study (Boy et al., 1990) involving 82 EPM cases, they found a higher incidence of disease in male horses. However, other studies (Bentz et al., 1997; Blythe et al., 1997; Saville et al., 1997; Tillotson et al., 1999; Vardeleon et al., 2001) were inconclusive with respect to gender bias.

Overall, the seroprevalence was correlated with the presence of the definitive host, the opossum. Environmental conditions (temperature, humidity) likely influenced the survival of sporocysts in the environment. There appeared to be some breeds which were predisposed to EPM based on some of the published papers. At this time, it is not clear if there is, and what the association is, with particular breeds. One other factor that may have predisposed horses to developing EPM was stress and associated induced immune suppression (Saville et al., 2000a; Saville et al., 2000b). Evidence for the latter was based on overall seroprevalence versus incidence of disease combined with reported risk factors. Despite the high seroprevalence (> 50% nationwide), only 0.5 – 1% of horses develop EPM based on a retrospective study of post mortem cases (Fayer et al., 1990). This would suggest that in most cases the host immune response was capable of preventing neurologic disease. Recently, Saville et al. (2000) identified several risk factors associated with EPM. These included competition, performance, injury, trauma, surgery, and parturition.

All of these factors were associated with stress. Therefore, a hypothesis was made that stressful events may cause an associated immune suppression, which subsequently increased the risk toward developing EPM. Furthermore, as *S. neurona*-infected immunocompetent mice were resistant to disease whereas some *S. neurona*-infected immunocompromised mice were susceptible to disease, this also suggested a critical role for immune system in protection against neurologic disease.

2.4 Experimental Animal Models

As establishment of an equine model has been challenging, both mice and horses have been widely employed as animal models in EPM research to help elucidate our knowledge of this disease. Cats, as intermediate hosts, have been used by some investigators to examine the life cycle of *S. neurona* and to also help explain the epidemiology of EPM (Butcher et al., 2002; Dubey et al., 2002; Stanek et al., 2003). As people realized the important role of the immune system in the pathogenesis of EPM, different immunodeficient mouse strains were utilized to help understand the influence of immune cell subsets and related cytokines on protecting horses from *S. neurona* infection and neurologic disease. The advantages of mouse models over an equine model included decreased costs in animal purchasing and housing, ease of handling and maintenance, extensively available immunodeficient strains, and murine specific reagents for immune function assays. However, one potential disadvantage of mouse models was the physiological differences between horses and mice which could possibly bias our understanding of both the protective and pathophysiologic immune response in horses.

In order to generate a mouse model which most closely mimics EPM in horses, considerable research employing different mouse strains, inoculation doses, routes of infection, *S. neurona* strains, and studies assessing *S. neurona*'s developmental stages were conducted (Marsh et al., 1997; Dubey and Lindsay, 1998; Cheadle et al., 2001; Dubey, 2001b, 2001c). One strain commonly used has been the interferon gamma knockout (GKO) mice. A minimum dose of 1,000 merozoites or more subcutaneously (s.c.) was necessary to consistently induce neurologic signs and seroconversion in GKO mice (Dubey et al., 2001b). Studies to determine the infective stage of *S. neurona* determined that both the merozoites and sporocysts were infectious to GKO mice through oral or s.c. inoculation (Dubey et al., 2001b). Long term storage (> 7 months) of *S. neurona* sporocysts could decrease their viability and a minimum dose of 500 sporocysts ($\leqslant$ 7 months) administered orally was required to produce consistent neurologic disease in GKO mice. Total dose, beyond the minimum dose, did not appear to affect severity of clinical signs, but did affect the time course of the disease based on oral infection (Cheadle et al., 2001). There was a dose dependent development of clinical signs of encephalitis in all infected GKO mice. The clinical signs in mice included paralysis, rough coat, eye squinting, mild to severe ataxia, head tilt, and circling (Cheadle et al., 2001). Parasitemia was detected at day 1 PI (post infection), when GKO mice were infected orally with sporocysts. *S. neurona* was first detected in the brain at day 4 – 9 PI (Dubey, 2001c). Other than the CNS, *S. neurona* was also primarily identified by immunohistochemical staining in the intestine, liver, and lung (Dubey, 2001c).

As GKO mice were susceptible to *S. neurona* infection, IFN-γ was crucial in protecting mice from disease (Sellon et al., 2004a; Sellon et al., 2004b). As we know, IFN-γ is secreted by a variety of cell types including CD8 T cells, Th1 CD4 cells, γδ T cells, NK cells, and other cells in the brain such as astrocytes, macrophages, dentritic cells, and microglia (Benveniste, 1998; Suzuki, 2002). One of the major functions of IFN-γ in modulating the innate response is to activate macrophages to kill intracellular pathogens through phagocytosis (Tizard, 2002). Although the protective immune response in horses has not been determined, a series of studies in mice have been performed to elucidate the role of important immune cell subsets like B-cells, CD4 T cells, CD8 T cells, and cytokines like IFN-γ in preventing *S. neurona* infection and development of disease (Marsh et al., 1997; Sellon et al., 2004a; Witonsky et al., 2003a, 2003b; Witonsky et al., 2005a; Witonsky et al., 2005b). Marsh et al. (1997) first found that C57BL/6 nude mice, not immunocompetent C57BL/6 mice or ICR severe combined immunodeficient (SCID) mice, developed encephalitis when inoculated intraperitoneally with culture-derived merozoites of *S. neurona*. However, GKO mice have been more commonly used in EPM research as a mouse model due to the savings in animal costs and less restricted environment required. In addition, GKO mice provide a unique way to investigate the role of IFN-γ in preventing *S. neurona* infection compared to nude mice. Both *S. neurona* infected nude mice and GKO mice develop encephalitis as do horses with EPM (Marsh et al., 1997). In the studies by Rosypal and colleagues, inducible nitric oxide synthase gene knockout (iNOS-KO) mice and endothelial nitric oxide synthase gene knockout (eNOS-KO) were resistant to *S. neurona*

infection, which suggested that IFN-γ mediated immunity to *S. neurona* may not be nitric oxide-dependent (Rosypal et al., 2002).

Immunopathgenesis studies on C57BL/6, GKO, and CD8 KO mice by Witonsky (Witonsky et al., 2003a, 2003b; Witonsky et al., 2005a; Witonsky et al., 2005b) found that CD8 T cells played an important role in preventing *S. neurona* infected mice from developing meningoencephalitis. B cell deficient mice (mμMT) did not seroconvert, nor did they develop encephalitis after inoculating them s.c. with *S. neurona* merozoites. This suggests that protection in mice to *S. neurona* infection was not mediated by B cell humoral immunity. Studies by Sellon et al. (2004a) on SCID mice depleted of NK cells provided further support for the role of IFN-γ. These studies showed that *S. neurona* infected SCID mice, which were also depleted of NK cells by Sellon, remained healthy following infection unless they received treatment with anti-IFN-γ antibodies. Once given IFN-γ antibodies, mice succumbed to encephalitis. In summary, these data suggested that the ideal protective immune response against *S. neurona* infection probably consists of both CD4 and CD8 cells as well as IFN-γ by multiple cell types for mice and horses.

Many studies have been performed in horses to try to develop a reliable model of EPM to investigate the pathogenesis of EPM. In 1997, Fenger et al. first successfully induced clinical signs consistent with EPM by feeding horses *S. neurona* and *S. falcatula* sporocysts, which had been shed by opossums (Dubey et al., 1991; Fenger et al., 1994). Only *S. neurona* sporocysts induced EPM. These horses developed antibodies to S. *neurona* in the serum and CSF as detected by

Western Blot (WB). Unfortunately, *S. neurona* was not identified by histopathologic analysis of the tissue sections. Subsequently, Cutter, Lindsay, Saville, Sellon, and Sofaly performed several experiments to develop a consistent model for EPM by varying *S. neurona* sporocyst dose, parasite strain, routes of inoculation, immune competency, and stress (Lindsay et al., 2000; Cutler et al., 2001; Saville et al., 2001; Sofaly et al., 2002; Saville et al., 2004a; Saville et al., 2004b; Sellon et al., 2004b). With the model utilized by Sofaly and Saville, the horses developed clinical signs, but not consistently in each study. However, Saville, Sofaly and colleagues were not able to isolate *S. neurona* parasites from the tissues of experimentally infected horses employing a combination of methods including light microscopy, immunohistochemistry, PCR, bovine turbinate cell culture, or bioassay in GKO mice. In a separate study, Long and colleagues, however, were able to isolate *S. neurona* from blood on an immunodeficient Arabian horse (Long et al., 2002). Unfortunately, this horse never developed clinical signs consistent with neurologic disease.

These experiments suggested that naturally infected horses could routinely prevent disease. Dexamethasone or transportation stress alone may be insufficient and/or less important than other factors in the development of EPM. Stress affects the immune response via secretion of neuroendocrine hormones (i.e. catecholamines), including dopamine, epinephrine, and norepinephrine (Cunningham, 1997). The sympathetic nervous system innervates primary and secondary lymphoid organs. In stressful conditions, the sympathetic nervous system sends action potentials to the synaptic cleft and neurotransmitters are released from

the presynaptic membrane. These neurotransmitters reach the cells in the immune system to influence the immune system through blood circulation or via direct binding to receptors on the postsynaptic membrane. Lymphocytes and monocytes express receptors for those stress hormones (Marshall and Agarwal, 2000). Experimental laboratory stressors on human subjects using speech and mental arithmetic tasks have been reported to suppress the *in vitro* NK cell activity; suppress mitogen-induced lymphocyte proliferation of T lymphocytes; lower the percentage of CD4 T helper cells in peripheral blood; and lower the CD4+/CD8+ T cell ratio (Knapp et al., 1992; Leonard, 1995). Stress events may either activate or suppress immune response depending on the cytokines produced by neuroendocrine mediator-activated immune cells. The exact mechanism of acute sympathetic-immune mediation is unclear.

Just recently, Sellon et al. (2004b) isolated the parasite from CNS tissue in immunocompetent (IC) horses, and visceral tissues only in severe combined immune deficiency (SCID) horses respectively. Again, the SCID horses did not show clinical signs of neurologic disease; these results were consistent with those by Long et al. (2002). Possible explanations for the lack of neurologic disease in SCID horses and mice include adequate protection mediated by innate immunity (i.e. NK cells, neutrophils, macrophages, DCs, microglial cells) and a lack of critical cell populations to cause disease. A previous study by Sellon et al. (2004a) on SCID mice demonstrated that IFN-γ protected SCID mice from developing neurologic signs. It is possible that the protection against *S. neurona* infection in SCID horses is mediated by IFN-γ produced by other cells like NK cells in the periphery and/or

CNS (Sellon et al., 2004b). Another possible explanation is that SCID mice and horses may lack certain immune populations (i.e. lymphocytes) which transport *S. neurona* sporocysts to the CNS. In IC horses, lymphocytes may facilitate *S. neurona* migration through the BBB to enter the CNS and cause damage. However, when IFN-γ function was blocked by IFN-γ antibodies, SCID mice, which were depleted of NK cells, developed neurologic signs (Sellon et al., 2004a). This suggested that under certain conditions *S. neurona* could migrate to the CNS, possibly through other leukocytes, which was not normally possible because IFN-γ activity prevented infection of critical cells. The latter explanation still needs to be tested.

One other model employing immunocompetent horses has been developed by Ellison and collegues (Ellison et al., 2004). In this model, peripheral blood lymphocytes were separated and infected with *S. neurona* merozoites. Then, each horse was inoculated intravenously with approximately 6000 *S. neurona* infected autologous leukocytes daily for 14 days. This model consistently induced clinical signs of neurologic disease and *S. neurona* antibodies specific to *S. neurona* in the CSF and serum. Histopathologic lesions were consistent to those in naturally occurring equine cases of EPM. *S. neurona* organisms were isolated from peripheral blood and CNS. This evidence indicated that Ellison's model was comparable in many ways to naturally infected horses of EPM. Another recent study by Rossano (Rossano et al., 2005) showed that parasitemia could be experimentally induced in *S. neurona* infected IC horses. Six IC horses were orally infected with 100 *S. neurona* sporocysts daily for 28 days (100 x 28 d), followed by 500 x 28 d, and finally 1000 x 56 d. In one horse, *S. neurona* was isolated from the peripheral blood, although none

of the horses developed disease. These data indicated, both the extreme conditions needed in order to induce a parasitemia, and the overall inherent protective immune response of the host associated with clearance of *S. neurona*.

In conclusion, the recent research on developing a reliable horse model for EPM has achieved remarkable progress. Future studies on horse models will focus on the role of immune response, particularly immune cell subsets and IFN-γ, in the pathogenesis of EPM.

2.5 Clinical signs

Clincal signs of EPM are predominantly associated with lesions in the nervous system. The clinical signs of EPM may be associated with either the body's immune response to the organism, or due to direct neuronal damage within the nervous system by the parasite *S. neurona* (Beech and Dodd, 1974; MacKay et al., 1992). The most common signs due to EPM include ataxia, asymmetry, and muscle atrophy; and these signs can either be focal or multi-focal. Other fairly common manifestations include lethargy, reduced exercise tolerance, lameness, which is not exacerbated by flexion, difficulty with complex gaits, and incoordination (Mackay, 2000). Additional central signs include head tilt, headshaking, dysphagia, and facial paralysis. The onset and development of clinical signs is variable with either sudden onset over hours or days to slow progression over several months. EPM-affected horses are commonly seen by veterinarians with a presenting/complaint of lameness, or failure to perform adequately (MacKay, 1997a).

The actual clinical signs of each case depend on the localization of *S. neurona* within the nervous system and periphery. If *S. neurona* localizes to the brain, the lesions may be consistent with encephalitis; whereas if the damage is in the spinal cord, this would be more consistent with myelitis. Lesions in the spinal cord are most common, but many infected horses have lesions in the brain as well (Mayhew, 1978; Fenger, 1997b). The clinical signs associated with the deficits are determined by the location of *S. neurona* within the nervous system. Damage to gray matter damage causes focal muscle atrophy and severe muscle weakness (paresis), while damage to the white matter often results in ataxia and weakness (paresis) in the limbs (Mackay, 1997a).

2.6 Diagnosis

EPM is one of the most common neurologic diseases in horses in the United States (Dubey et al., 2001a). When a horse develops abnormal neurologic signs consistent with EPM, the latter should almost always be considered as a possible diagnosis (Mackay et al., 2000). However, a diagnosis by clinical signs alone is difficult because other diseases such as equine degenerative myelopathy, equine herpes virus myelitis, muscloskeletal disorders, and West Nile virus, can also cause similar neurologic signs. A veterinarian should first conduct a thorough physical and neurologic examination to assess the horse's general health and identify any signs consistent with EPM versus signs consistent with other causes of neurologic disease.

Presently, one of the most common ways to diagnose EPM premortem is based on clinical signs consistent with EPM and Western Blot to detect antibodies to *S. neurona* in CSF and serum. A seropositive test alone just indicates that the horse has been exposed to the parasite, not that it has or will develop clinical disease. Some of the different diagnostic assays will be mentioned here, but they will be discussed in more detail subsequently. PCR testing of equine CSF can detect the presence of *S. neurona* DNA in the CNS (Fenger et al., 1994; Granstrom et al., 1994; Marsh et al., 1996b). However, the specificity is low because of the high number of false negative results for disease detection. Other than *S. neurona* specific diagnostic tests, additional diagnostics for suspect EPM horses often include complete blood cell counts and clinical serum biochemistry tests. These can be used to exclude other causes of neurologic diseases (i.e. liver, renal disease), but often there are no abnormal findings on EPM cases (Mackay, 1997a).

2.6.1 Cytology and Cerebrospinal Fluid Indices (CSF indices)

Once CSF samples are obtained from suspect EPM horses, analysis often consists of cytology, protein analysis, WB, and CSF indices. Cytology is performed to examine the cells present (i.e. neutrophils, lymphocytes, RBC), to determine if there is an increase in one or more immune cell subsets, and to determine if the cytology is consistent with EPM (i.e. mononuclear cells) vs other neurologic diseases (i.e. West Nile). An increase in RBC's can indicate contamination, trauma, or vascular damage. An elevated protein level can be due to contamination, EPM, or other neurologic diseases. Samples are analyzed for *S. neurona* by one or more of

the following: WB, IFA, PCR, and ELISA. Finally, CSF indices are also calculated. The indices provide an indication of intrathecal antibody production. Also, CSF indices provide information regarding the integrity of the BBB. Collectively this information is used to interpret the results of WB, IFA, PCR, and/or ELISA. When CSF is contaminated with peripheral blood or the BBB is breached, serum antibodies can leak into the CSF and cause false positive results for *S. neurona* antibodies in the CSF (Dubey et al., 2001a; Miller et al., 1999). Cytologic examination of CSF samples can reveal that as few as 8 RBCs / mm^{-3} in the CSF can indicate enough damage to the BBB or contamination to cause a false positive result (Miller et al., 1999).

To calculate CSF indices, it is necessary to determine the total IgG concentration in serum and CSF, and the albumin concentration in serum and CSF. CSF indices are in the form of albumin quotient (AQ) and IgG index. The AQ is the ratio of CSF albumin to serum albumin (Figure 2.3). An elevated AQ may indicate increased BBB permeability or accidental blood contamination of the sample. The IgG index is calculated by dividing the amount of CSF IgG by serum IgG, and taking the AQ into account (Figure 2.3) (Andrews et al., 1994). An increased IgG index is an indicator of intrathecal antibody production. Since the increase in IgG may be due to other diseases resembling EPM, CSF indices must be interpreted with caution (Cohen and MacKay, 1997; Miller et al., 1999).

Albumin Quotient (AQ) = Albumin CSF /Albumin serum X 100

(Normal range is 1.0 to 2.0)

IgG index = IgG CSF / IgG serum X AQ

(Normal range is 0.1 to 0.3)

Fig 2.3: CSF-indices (Cook, 2001).

2.6.2 Enzyme-Linked Immunosorbent Assay (ELISA) to *Sarcocystis neurona* Surface Antigen 1 (SnSAG1)

An ELISA to SnSAG1 has recently been developed to detect serum and CSF antibodies to *S. neurona* specific antigen SnSAG1 (Ellison et al., 2005). After a 29 kDa surface antigen from *S. neurona* merozoites was identified as immunodominant, the antigen was cloned, and sequenced. The expressed protein was named SnSAG1 (Ellison et al., 2002). In this assay, SnSAG-1 was utilized to detect *S. neurona* SAG-1 antibodies in the serum or CSF from infected horses. In the WB, whole *S. neurona* merozoites were used as antigens to detect antibodies, which could have caused some false positive results. Furthermore, the WB was performed under denaturing conditions, which have been shown to decrease the ability to detect SAG-1 antibodies (Ellison et al., 2004). In addition, SnSAG-1 ELISA provided quantitative information about *S. neurona* antibodies in infected horses, but the WB only indicated qualitative results (i.e. positive, low positive, weak positive, or negative). The formula for how to calculate a specific antibody index (AI_{SAG1}) utilizing the ELISA is in Figure 2.4.

One potential limitation of the SnSAG-1 may be the ability to detect *S. neurona* infected horses, in which the *S. neurona* strain lacks SnSAG-1. Data by others (Dan Howe, personal communication) suggests that some strains may lack SnSAG-1. These data must be confirmed. If accurate, and there is not any cross-reactivity between other SnSAG proteins and SnSAG-1, this would be a limitation of the SnSAG-1 ELISA.

AQ = (CSF SAG-1 titer) X 1000/ (serum SAG-1 titer)

AlbQ = (CSF alb/serum alb) X 1000

AI_{SAG1} = AQ/AlbQ

AQ: antibody quotient

AlbQ: albumin quotient

Titer: the reciprocal of the last dilution with a positive $_{A405}$.

Fig 2.4: Calculation of AI SAG1 index (Furr et al., 2002)

2.6.3 Immunoblot assay (western blot)

The immunoblot assay for *S. neurona* was also called the Western Blot (WB). This technique was employed to identify the presence of *S. neurona* specific antibodies in serum and CSF (Granstrom et al., 1993). It was developed in 1991 using cultured *S. neurona* merozoites from the spinal cord of an EPM horse (Davis et al., 1991; Granstrom et al., 1992) to examine sera and CSF from suspect EPM horses. Typically, *S. neurona* antigens were separated by molecular weight via gel electrophoresis. The separated antigens were then transferred to a supportive membrane. Serum or CSF samples from suspect or control horses were then incubated with the membrane. Samples were developed with alkaline phosphatase. In the studies by Granstrom, Marsh, and Liang, eight specific proteins were recognized by antibodies produced in either horses with EPM or horses and rabbits injected with *S. neurona* (Granstrom et al., 1993; Marsh et al., 1996b; Liang et al., 1998). Currently, at least three different commercial laboratories (IDEXX Inc., University of Kentucky, Michigan State University) have provided an immunoblot assay for EPM testing. Their differences in methodology were slightly different, and their criteria for a positive result was different based on exclusion of certain antibodies to particular antigens. A positive WB result indicated the presence of antigen specific antibodies, but it did not quantitate the amount of antibody present. One additional disadvantage of the WB, as performed by IDEXX, was that it was performed under denaturing conditions. As stated before, this approach has been demonstrated to specifically decrease the ability to detect antibodies to SAG-1

(Ellison et al., 2004). Thus, it can cause false negative results under certain conditions.

2.6.4 Immunofluorescence Assay (IFA)

IFA is another reliable way to diagnose EPM with serum and CSF (Duarte et al., 2003, 2004). By comparison to WB tests, the IFA test can provide information on the amount of the antibody present based on a titer. IFA is currently run utilizing the protocol described by Vardeleon et al. (2001). A difference between the WB and IFA was that with the IFA, horse serum or CSF was incubated with prepared *S. neurona* merozoite antigen. After the first incubation and wash, a second fluorescein-labeled affinity purified anti-equine IgG was added. Following incubation and washing, the samples were read utilizing a fluorescence microscope.

2.6.5 Serum Agglutination Test (SAT)

SAT was developed by Lindsay and Dubey (2001) to detect *S. neurona* antibodies in experimentally infected animals (Lindsay and Dubey, 2001). In this test, merozoites were washed and resuspended in alkaline buffer-eosin solution. IgM antibodies were destroyed by B-mercaptoethanol to prevent non-specific agglutination reaction. Briefly, merozoite solution was coated in 96-well round bottom plates. Then, 25 µl of serial dilution of serum or CSF was added and mixed well before overnight incubation. A positive result was recorded if the diffuse opacity crossed the entire diameter of the well. The sensitivity and specificity of the

SAT in mice was 100 and 90%, respectively based on the study by Lindsay and Dubey. Lindsay has also employed this assay utilizing serum and CSF from naturally infected horses (unpublished data).

2.6.6 Polymerase Chain Reaction (PCR)

A DNA test has also been developed for detecting *S. neurona* infection in the central nervous system of horses (Fenger et al., 1994). The big advantage of employing PCR as a diagnostic test for EPM was that *S. neurona* could be detected in the early stages of infection before the host immune response produced antibodies in CNS. In fact, there have been cases which test positive for *S. neurona* DNA but are negative for antibodies to *S. neurona* in the CSF (Miller et al., 1999). However, the sensitivity and specificity of this test are not optimal. The limitations of the assay are that there are a lot of false negatives for disease. This is because *S. neurona* is an intracellular organism, and is rarely thought to be present in the CSF and/or because *S. neurona* DNA in the CSF is probably degraded rapidly by enzymatic activity (Marsh et al., 1996b). Therefore, a positive PCR result is very specific, but due to limitations in sensitivity of the test to detect a horse with EPM, the PCR test is a useful supplemental, but not exclusive test in the diagnosis of selected cases of EPM.

2.7 Pathology

Horses with EPM may not have gross abnormalities on necropsy (Fenger, 1997b). If lesions are present, the spinal cord is the most affected tissue in the CNS (Fayer et

al., 1990; Fenger et al., 1994). Destruction caused by *S. neurona* infection has never been reported in tissues outside the CNS in IC horses (Cook, 2001; Dubey et al., 2001a). Lumbar and cervical regions may be the most commonly affected locations within the spinal cord (Rooney et al., 1970; Cook, 2001). Histopathology can be employed to grade the chronicity of disease. Acute lesions exhibit multifocal randomly distributed hemorrhage, prominent perivascular cuffing of lymphocytes, and minimal tissue destruction. In chronic cases, lesions include astrocytosis and gliosis with loss of neuronal structure, or phagocytes ingesting myelin in white matter regions (Mayhew et al., 1978; Cook, 2001). The inflammatory response is highly variable in cases and has consisted of a mixture of lymphocytes, neutrophils, eosinophils, multinucleate giant cells, and gitter cells (Dubey et al., 2001a).

Despite the histopathologic changes present, detection of *S. neurona* in the lesions has been rare. A definite explanation is lacking. Possible explanations include the fact that *S. neurona* remains intracellular, which may decrease the chance of detection; that the number of organisms may be beyond that easily detected by routine histopathology and/or immunohistochemistry, and/or that *S. neurona* caused initial persistent damage, but is not present at the time of necropsy (Bowman et al., 1992; Dubey et al., 1991, Cook, 2001).

2.8 Treatment

Therapy for horses affected with EPM includes several different strategies. The most important and common therapeutic includes specific “anti-protozoal” drugs

that inhibit protozoal replication (Dubey et al., 2001a). Second, anti-inflammatory medications, such as flunixin meglumine, are often administered, and they serve to inhibit inflammation and swelling systemically (Dubey et al., 2001a). Supportive care and supplemental treatment of any secondary problems may also be necessary. Often these horses may develop pressure sores which need treatment, or in some situations, EPM affected horses can become dysphagic and need additional support (Mackay, 2000; Dubey et al., 2001).

The drug combination of pyrimethamine and sulfadiazine is the most traditional treatment for EPM. Pyrimethamine is an anti-protozoal drug and sulfadiazine is a sulfa antibiotic. The combination of these drugs has a synergistic effect against *S. neurona* (Fenger et al., 1997b). Each drug blocks a different step in the metabolism of the protozoa by inhibiting dihydropteroate synthase necessary for the synthesis of folic acid (Colahan et al., 2002). This drug combination could either kill the protozoa or inhibit additional growth or reproduction depending on the dose (Lindsay et al., 1999). Neither drug alone is considered effective for the treatment of EPM (Fenger et al., 1997b). Furthermore, horses treated with pyrimethamine/sulfadiazine often require long-term treatment (12 to 24 weeks or longer) which also has the potential to inhibit folic acid metabolism in the horse. These side effects include anemia, decreased platelets, decreased white blood cell counts, and bone marrow suppression (Cook, 2001). Because of these potential side effects, periodic monitoring of complete blood counts is recommended with this treatment; horses are supplemented with folic acid when necessary.

Currently, there are two new medications for the treatment of EPM. In July of 2001, the FDA approved Marquis antiprotozoal oral paste, the first medication for the treatment of EPM in the United States. The active ingredient of Marquis is ponazuril, an anticoccidial compound with dose dependent cidal activity against *S. neurona*. While Marquis could possibly effectively eliminate *S. neurona* infection, it will have no effect on irreparable, central nervous system damage caused by the parasite prior to treatment (Furr et al., 2001). Another new option for treating EPM is nitazoxanide, which was marketed by Idexx and approved by the FDA in 2003. The antiprotozoal activity of nitazoxanide (NTZ) is believed to be due to interference with the pyruvate ferredoxin oxidoreductase (PFOR) enzyme-dependent electron transfer reaction, which is essential to anaerobic energy metabolism (Lindsay et al., 1998; McClure, 1999). One open field study by IDEXX company showed that it treated 81% of more than 400 horses within the study. In this same study, 78% of horses that had been previously treated unsuccessfully for EPM were successfully after receiving this treatment (http://www.idexx.com/equine/pharmaceuticals/navigator/index.jsp).

2.9 Prevention

Prevention of EPM becomes extremely important because of the limited efficacy of current treatments. The best, although very challenging, way to try to reduce incidence of EPM is to limit the access of opossums, the definitive host of *S. neurona*, and other wildlife or pests, which may be intermediate hosts or may spread *S. neurona* infected feces, to horse feed, pasture, and water. Since opossums are

nocturnal omnivores and scavengers, horse feed and pet food should be kept in closed containers. It is advisable to keep birds away from the farms because their feces may contain *S. neurona* sporocyts (Cook, 2001; Mackay et al., 2000).

An efficacious vaccine would be an effective way to prevent horses from developing EPM due to *S. neurona* infection. However, there have been many difficulties in developing an effective protozoal vaccine (Cook, 2001). *S. neurona* is an intracellular organism, which must be attacked by components of the cell-mediated branch of the immune system, such as by cytotoxic T lymphocytes. Most vaccines do not work well in eliciting cell-mediated immunity. Another problem is that protozoa (i.e. *Trypanosomes brucei*) can evade the host's immune system by changing its surface antigens during development (Tizard, 2002). In 2000, Fort Dodge Animal Health (Fort Dodge, IA) developed a crude vaccine from lysates of whole cultured *S. neurona* merozoites. Studies by Marsh et al. (2004) with the same killed *S. neurona* merozoite vaccine demonstrated that clinically normal horses developed *in vitro* and *in vivo* cell-mediated immunity (CMI) to *S. neurona* merozoite antigens following IM injection (Marsh et al., 2004). Challenge studies have been performed with the same vaccine. However, the data have not been released (Ellison, personal commmunication).

2.10 Future goals

Many studies utilizing horses have been undertaken to investigate the nature of the protective immune response to *S. neurona* infection. Tornquist and colleagues

reported decreased cell-mediated immune responses to T-cell mitogen concanavalin A (ConA) and *S. neurona* antigen in naturally occurring cases of EPM (Tornquist et al., 2001). Spencer and colleagues reported that lymphocytes from *S. neurona* sero-negative horses had greater proliferation responses to SnSAG-1 than lymphocytes from EPM cases (sero-positive horses with neurologic disease consistent with EPM) (Spencer et al., 2004). IFN-γ production in response to SnSAG-1 was decreased in EPM-positive horses (Spencer et al., 2004). The above studies support the notion that *S. neurona* merozoites are able to suppress specific immune responses directed toward this parasite. Further studies by Spencer et al. (2005) indicated that the immunosuppression was at least partly due to suppressed Th1 cytokine gene expression (IFN-γ) and increased Th2 cytokine gene expression (IL-4).

Future studies are expected to focus on developing an efficacious vaccine, understanding the pathophysiology of disease, and developing more sensitive diagnostic assays to decrease losses due to EPM. In trying to develop a protective vaccine, efforts have focused on identifying putative immunodominant and possibly protective antigens. Liang et al. reported that antibodies to two surface proteins Sn14 and Sn16 of *S. neurona* inhibited merozoite growth, which suggests that these two proteins could reduce parasite infection by binding to the merozoite cell surface and further preventing merozoites from attaching to or penetrating host cells (Liang et al., 1998). More recently, in an effort to develop a more sensitive and possibly specific assay, Gupta (Gupta et al., 2004) developed a WB utilizing only a 29 kDa *S. neurona* surface antigen (rSnSAG1-Bac). Results with this WB were comparable to those utilizing whole *S. neurona* merozoite lysates. Use of this recombinant surface

antigen SnSAG-1 in diagnostic test has the potential to replace the traditional WB. Although the 29 kDa SnSAG protein is thought to be immunodominant, further studies are needed to demonstrate whether it is protective. If so, it could be used as a candidate protein for a vaccine.

CHAPTER 3 Immunophenotypes and lymphocyte proliferative responses in naturally infected EPM horses.

3.1 Introduction

Equine protozoal myeloencephalitis (EPM) is caused by a protozoal infection which affects the central nervous system (Rooney et al., 1970). It is one of the most common neurologic diseases of horses in North America (MacKay, 1997a). Approximately 50% of horses in the US are seropositive to *S. neurona* (MacKay, 1997b, 2000). However, only a minority of all *S. neurona* seropositive horses actually develop clinical signs of neurologic disease (Cohen and MacKay, 1997). It is possible that seropositive horses with neurologic signs could have been immunocompromised due to *S. neurona* infection or pre-existing immunosuppression prior to infection. In an experimental model, horses exposed to transportation stress immediately before infection were likely to develop more severe neurologic signs (Saville et al., 2001), which suggested that stress could have induced immunosuppression and decreased the ability of *S. neurona* challenged horses to protect against *S. neurona* mediated clinical disease.

Based on the above observations we hypothesized that horses with EPM have altered immune responses compared to neurologically normal seropositive horses. More specifically, we were interested in investigating if the immunosuppresion in EPM horses was due to a decreased cell-mediated immune response. The main T-cell immune cell subpopulations CD4 and CD8 were measured, and IFN-γ

production between experimentally infected horses and normal horses stimulated with non-antigen specific and antigen specific mitogens was compared. For these experiments, the percentages of CD4, CD8, B-cells, monocytes and neutrophils in peripheral blood were measured between clinically normal and EPM horses. Changes in immune function were investigated employing lymphoblastogenesis assays to assess antigen specific and non-specific function.

3.2 Materials and methods

3.2.1 Animals: Forty-two horses of various breeds were included in the study. The twenty-two EPM horses were either presented to the Veterinary Teaching Hospital of Virginia-Maryland Regional College of Veterinary Medicine (VMRCVM), donated by individual clients, or were seen by an ambulatory veterinarian on the farm. Horses were a combination of mares, stallions, and geldings ranging from 1-20 years of age. Their breeds consisted of predominantly Thoroughbred, Quarter horse, Arab, warmblood, or mixed breed. Horses were diagnosed with EPM based on characteristic clinical signs, detection of *S. neurona* antibodies in the serum and/or CSF (Granstrom 1995) by WB, and/or SAG-1 analysis and response to treatment. Western blot analysis was performed at a commercial laboratory (EBI/Idexx, Lexington, KY). SAG-1 analysis was performed by Ellison (Pathogenes, Fairfield, FL). Twenty neurologically normal female horses from Veterinary Teaching Herd of VMRCVM were utilized as controls. Their ages ranged from 6 to 23 years. Breeds consisted of Thoroughbreds (7), Quarterhorses (3), Rocky Mountain, Arab, Warmblood, and mixed breed horses. All protocols and

procedures were approved by the Institutional Animal Care and Use Committee at Virginia Polytechnic Institute and State University.

3.2.2 Collection of blood samples: With respect to sampling, when possible, blood was collected, divided in half, and half the blood was analyzed immediately (day 0) and half the blood was refrigerated overnight and analyzed the following day (day 1). Blood from control horses was collected and stored similarly. When blood was either shipped or collected late in the day, blood from control horses was also collected simultaneously. All blood samples were shipped with cold packs overnight and control blood was stored overnight in the refrigerator. Samples were run on the following day (day 1 samples only). From each horse, approximately 30-40 ml of peripheral venous blood was collected in lithium heparinized tubes (Vacutainer tubes, Fisher Scientific, Suwanee, GA) by aseptic venipuncture from either the left or right jugular vein. When possible blood smears were made immediately following collection and used to assess changes in immune subset viability followed overnight incubation. All the stored or shipped blood was warmed up to room temperature (23 ^{0}C ± 2) before evaluation (Witonsky et al., 2003c). Blood smears were made to assess viability. PBMCs were isolated using a modification of previously described methods (Witonsky et al., 2003c).

3.2.3 Differential cell counts: Five point - differentials were performed on each blood sample to determine the percentage of each leukocyte subset. Slides were stained with modified Wright stain (Modified Wright stain, Sigma Chemical Co, St

Louis, Mo). One hundred cells were randomly counted to determine the differential (Witonsky et al., 2003c).

3.2.4 Isolation of Peripheral Blood Mononuclear Cells (PBMCs): PBMCs were isolated by density gradient centrifugation (Witonsky et al., 2003c). Whole blood at room temperature was diluted with Phosphate Buffered Saline (PBS, Mediatech, Herndon, VA) at the ratio of 1:2 before carefully layering the diluted blood (2:1) on Lymphoprep (Lymphoprep 1.077, Greine, NJ). In our experience, all these reagents must be at room temperature for optimal purity of lymphocytes, otherwise there can be considerable neutrophil contamination (unpublished data). Samples were centrifuged at 1,500 rpm (350 x g) for 30 min at 23 ^{0}C without the brake. The buffy coat was collected and diluted with minimally an equal volume of PBS. Cells were centrifuged at 1,300 rpm (250 x g) for 10 min at 4 ^{0}C. The supernatant was removed and samples were resuspended in PBS, and washed twice, spinning at 1,300 rpm (250 x g) for 5 min at 4 ^{0}C. Cell counts were determined with the CASY-1 (TTC cell counter and analyzer system, Sharfe System, GMbH, Reutingen, Germany). Cells were resuspended to a final concentration of 2 x 10^6 / ml in complete RPMI 1640 (Cellgro^{TM} RPMI 1640 1x, Thomas Scientific, Herndon, VA) with L-glutamine, Hepes Buffer 25 mM, 10% heat inactivated fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), penicillin (50 IU/ml), and streptomycin (50 IU/ml) (Sigma Chemical Co, St Louis, MO).

3.2.5 Cytospin preparation: Cells (0.25 x 10^6 cells) were diluted with 100 µl PBS and loaded to a centrifugation chamber. Each chamber was placed into a

cytocentrifuge (Cyto-Teck centrifuge, Sakura Finetechnical Co, Tokyo, Japan), and spun at 500 rpm (30 x g) for 5 min. The slides were air-dried followed by fixing and staining with modified Wright stain. Cover slips were placed, and 100 cells were enumerated and described as the percentages of lymphocytes, neutrophils, macrophages, basophils, or eosinophils (Witonsky et al., 2003c).

3.2.6 Flow cytometry: For each sample, 5 x 10^5 cells were placed in a 96 well round bottom plate (Corning Glass Works, Corning NY). Cells were washed with 4 ^{0}C PBS and centrifuged at 1,300 rpm (250g) for 5 min at 4 ^{0}C. The following monoclonal antibodies were added to individual samples: CD4 (mouse anti-equine CD4 antibody, cell line HB61A IgG_1, VMRD, Pullman, WA), CD8 (mouse anti-equine CD8 antibody, cell line HT14A, IgG_1 VMRD, Pullman, WA), B-cell (mouse anti-equine CD5 antibody, cell line B29A, IgG_{2a}, VMRD, Pullman, WA), and DH59b antibody (mouse anti-equine IgG_1, VMRD, Pullman, WA) to equine granulocytes. Cells were incubated with 0.5 μg of the primary antibody for 20 min at 4 ^{0}C. Cells were washed, and then incubated for 20 min at 4 ^{0}C with 0.5 μg secondary antibody of either FITC rat anti-mouse IgG_1 (for conjugation to CD4, CD8, and DH59b antibodies) (Pharmingen, San Diego, CA) or PE rat anti-mouse IgG_{2a} (for conjugation to B-cell antibody) (Pharmingen, San Diego, CA) at a concentration of 1 μg/10^6 cells. Unstained samples were incubated with PBS. Cells were washed and resuspended in 200 μl PBS (4 ^{0}C) for flow cytometry (EPICS XL flow cytometer, Coulter, Hialeah, FL). 7-AAD (as described below) was added prior to running the samples on the flow cytometer.

3.2.7 7-amino actinomycin D (7-AAD) staining: 7-AAD is a fluorescent DNA binding agent widely used for measuring apoptosis of cells. This dye intercalates between cytosine and guanine bases in the di-nucleotide strands and makes the cells fluoresce (Schmid et al., 1992). It is efficiently excluded by intact cells. The degree of binding depends on the extent of cell damage and permeability of the cell membrane. In addition, the dye can be used in combination with FITC or PE since its emission in the far red can easily be separated from the emission of FITC and PE fluorochromes. As the staining intensities vary between early apoptotic and late apoptotic/necrotic populations, flow cytometry can be utilized to distinguish between live, early, and late apoptotic/necrotic cell populations (Schmid I et al., 1992). In this study, it was used to detect the viability of PBMCs which were isolated from samples at day 0 and day 1.

After the samples were stained with the primary and secondary antibodies, followed by washing and resuspending the samples in PBS, 1 μg of 7-AAD (Molecular Probes, Eugene, OR) was added to each sample. The plate was incubated for no more than 30 min on ice in the dark. Five or ten thousand cells gated events per sample were collected by the flow cytometer. Based on the intensity of staining, cells were classified by their subset as $7AAD^{dull}$ (live cells), $7AAD^{moderate}$ (early apoptosis), and $7AAD^{bright}$ (late apoptosis) (Gogal et al., 2000).

3.2.8 Live merozoite preparation: *S. neurona* merozoites (SN-37R) (Sofaly et al., 2002) were grown and maintained in African green monkey (*Cercopithecus aethiops*) kidney cells (CV-1 cells, ATTC CCL-70, American Type Culture Collection,

Rockville, MD, USA). *S. neurona* merozoites were harvested from CV-1 cells by removing the complete media (RPMI with L-glutamine, hepes buffer 25 mM, 2% heat inactivated FBS, 50 IU/ml of penicillin/streptomycin solution, 1% sodium pyruvate solution, Mediatech, Herndon, VA). The suspension was filtered through a 3 μM filter and spun at 1,500 rpm (350 x g) for 10 min at room temperature, and then resuspended in complete media. The merozoites were enumerated with a hemacytometer and resuspended at a concentration of 1 x 10^5 /ml with complete media containing 10% heat inactivated FBS (Lindsay et al., 2000).

3.2.9 Lymphocyte Proliferation Assays: These assays were used to determine both whether there were mitogen-stimulated (non-antigen specific) and parasite-specific (antigen specific) differences in lymphocyte proliferation between normal and EPM horses, as well as to determine if particular subsets (i.e. T vs B-cells) were affected. The following different mitogens were used to stimulate different lymphocyte subpopulations. Concanavalin (ConA) stimulated equine T lymphocytes. Pokeweed mitogen (PWM) stimulated equine B lymphocytes. Phorbol Myristate Acetate (PMA) and ionomycin stimulated all leukocytes (Witonsky et al., 2003c). For antigen specific blastogenesis, live merozoites as well as purified recombinant *S. neurona* surface antigen-1 (SAG-1) were used as mitogens.

A 100 ul aliquot of equine enriched lymphocytes (2 x 10^6/ml) was cultured in triplicate wells together with 100 ul of the appropriate mitogen or live merozoites (1 x 10^5/ml) (Witonsky et al., 2003c). Final concentrations of mitogens in the wells were 1 μg/ml or 5 μg/ml ConA (Sigma Chemical Co, St Louis, MO), 1 μg/ml PWM

(Sigma Chemical Co, St Louis, MO), 20 ng/ml PMA and 10 pg/ml ionomycin (Sigma Chemical Co, St Louis, MO). Cells were incubated at 37 ^{0}C in humidified 5% CO_2 and pulsed with 1 μCi ^{3}H-thymidine after 48 hours. Plates were harvested 18-24 hours later using a Filtermake Harvester (Packard Bioscience, Billerica, MA). Delta counts per minute (ΔCPM) were calculated based on the mean CPM of cells with mitogens minus mean CPM of cells from unstimulated cells in media (spontaneous proliferation).

3.2.10 SnSAG-1 (Sarcocystis neurona surface antigen-1) plate incubation: A 29 kDa *S. neurona* surface antigen was characterized as an immunodominant protein (Ellison et al., 2002). The gene of this 29 kDa protein was cloned, and expressed as a recombinant protein named SnSAG1, which was used in the blastogenesis assay to determine the antigen-specific lymphocyte proliferation between EPM horses and normal horses. SnSAG1 was plated as described previously (Spencer et al., 2004). Briefly, 96-well round bottom plates were coated with 50 μl SnSAG1 (10 μg/ml) in PBS and incubated overnight at 4 ^{0}C. The plate was washed with PBS three times and 100 μl enriched lymphocytes (2 x 10^{6}/ml) plus 100 μl of complete media were added. Wells with cells only without SnSAG1 coating were used as negative controls. Cells stimulated by ConA (5 μg/ml) were positive controls. The plate was incubated 72 hours before adding ^{3}H-thymidine. After that, cells were incubated for another 18 to 24 hours and then harvested with Filtermake Harvester as described above.

3.3 Statistical Analysis

Analysis of variance was conducted using the GLM procedure of the SAS system (version 8.2, SAS Institute Inc., Cary, NC) to test for treatment effects and covariate, unless otherwise stated. Standard residual plots were used to assess model adequacy. Tukey-Kramer t test was used to establish differences. Results were expressed as mean ± SEM. The actual numbers of EPM and normal horses in each result may be different due to limited data collection of some samples or the statistical model.

3.4 Results

3.4.1 Effects of storage or shipping condition on immunological assays utilizing equine PBMCs: In order to identify the effect of storage or overnight shipping, the percentages of CD4 cells, CD8 cells, B-cells, monocytes, and neutrophils in peripheral blood were compared between day 0 and day 1 samples. There were no statistically significant differences in the immune cell subsets, except CD8 cells. The percentage of CD8 cells of total lymphocytes decreased significantly from 17.4 ± 1.5 on day 0 to 11.6 ± 1.5 on day 1 ($p < 0.0001$, Figure 3.1). Results are summarized in Table 3.1.

The cell viability, which was detected by 7-AAD, indicated that more than 90% of CD4 and CD8 cells were viable at both day 0 and day 1. Specific statisitical analysis was not performed on day 0 vs day 1 to determine whether there was an effect of day on viability. The percentage of viable B-cells ranged from 63% to 88%. No obvious differences appeared in cell viability associated with overnight shipping

or storage. Blood smear slides did not show any differences between samples in day 0 and day 1. Cytology data indicated that samples at day 1 tended to include more (1%) neutrophils compared to samples at day 0. Statistical analysis of cytology data was not performed.

Statistical results of lymphocyte proliferation assays are presented in Table 3.2. Notably, the storage or shipping condition significantly changed the proliferation responses stimulated by PWM and live *S. neurona* merozoites on day 1. Proliferation responses stimulated by PWM significantly increased from 21,485 ± 4,896.75 CPM on day 0 to 33,502 ± 5,006.59 CPM on day 1 when the results from both nomal and EPM horses were averaged by day to assess the effect of day (p = 0.0067, Figure 3.2). There was also a significant effect of day and day by treatment status on lymphocytes stimulated with *S. neurona* merozoites. However, the proliferation response trends in normal horses and EPM horses were different. The proliferation response in normal horses did not change significantly from day 0 to day 1. Day 1 lymphocytes in EPM horses had significantly higher proliferation responses to *S. neurona* merozoites than day 0 lymphocytes ($p < 0.0495$). Delta CPM in EPM horses increased from 1,451.73 ± 874.48 CPM in day 0 to 3,829.03 ± 947.18 CPM in day 1 (Figure 3.3). Statistical analysis did not compare normal horses to EPM horses on both day 0 and day 1.

Based on Casy data (assessment of cell numbers based on cell size) and cytology data, PMA/I stimulated proliferation responses were decreased if PBMCs were contaminated by neutrophils. Neutrophil contamination was an effect of temperature

(i.e. blood shipping at less than 4^0C or use of cold reagents increased the percentage of neutrophils recovered). Therefore, we employed the neutrophil as a covariate in the statistical analysis to increase the power of test for hypothesis. Results from SAG-1 antigen-specific stimulated proliferation response were not included due to very limited data for statistical analysis.

3.4.2 Analysis of immune cell subsets and changes in immune function between naturally infected EPM and clinically normal horses: All samples were divided into either day 0 and day 1 samples or day 1 samples only. In day 0 samples, 8 EPM and 8 matched normal horses were included. In day 1 samples, 15 EPM and 15 matched normal horses were collected. Eight of the 15 pairs for day 1 are the same horses as those in day 0.

Naturally infected horses had a significantly (p = 0.0163) higher percentage of CD4 cells (68.4% ± 3.9 vs 63% ± 3.9) than normal horses at day 0. The percentage of CD4 lymphocytes was still increased in EPM vs normal horses at day 1, but it was not significantly different (66.4% ± 3.17 in EPM horses vs 64.9% ± 3.17 in normal horses, Figure 3.4). The percentage of PMN leukocytes in EPM horses (7.50% ± 0.85) was significantly higher than in normal horses (5.15% ± 0.85) in day 0 samples only (p = 0.012).

There were no significant differences in leukocyte responses to ConA or PWM. For EPM samples on both day 0 and day 1, the proliferation responses stimulated by PMA/Ionomycin were lower than normal horses (Figure 3.6). The difference was

statistically significant at day 1 (p = 0.0035) and not significant at day 0 (p = 0.1298). There were no statistical differences in merozoite stimulated proliferation responses at both day 0 and day 1 samples between EPM and normal horses (Figure 3.7).

3.5 Discussion

These data were the first to evaluate the effect of shipping and/or storage conditions on equine immune function between EPM and normal horse blood samples. Witonsky et al. (2003c) tested whether equine blood samples stored overnight in a refrigerator could accurately be used to assess cellular immune function. It was found that the percentage of CD8 cells in lymphocytes decreased with storage, which was consistent with results obtained in this study. In blastogensis assays, Witonsky et al. (2003c) observed a significant decrease in ConA stimulated proliferation response in samples at 24 hours, compared to samples at 0 hours. In contrast, we did not see this difference in ConA stimulated response. Witonsky et al. (2003c) demonstrated that *Eschericheria coli* lipopolysaccharide (LPS) stimulated equine lymphocyte proliferation was weak compared to the proliferation induced by ConA or PMA/I. In our study, we employed PWM to detect B-cell immune function, and we found that PWM stimulated responses were increased in day 1 vs day 0 samples in both normal and EPM horses. This could be due to the increased percentage of B-cells in PBMCs at day 1 or altered B-cell proliferation rate by storage or shipping procedures. The flow cytometry did demonstrate non-statistically significant but elevated B-cell percentages in PBMCs

at day 1 vs day 0 (14.2 ± 1.765% vs 12.4 ± 1.817%). It was possible that this difference could have been physiologically significant.

With regard to antigen specific responses, our results were not entirely consistent with other studies (Tornquist et al., 2001; Spencer et al., 2004). In the Tornquist study in 2001, all the samples were presumably at day 0, and the *S. neurona* antigens were solubilized merozoites of the SN6 strain. Spencer's studies included both day 0 and day 1 samples, which were combined in the analysis. The *S. neurona* antigen was SnSAG-1. Similar to the studies by Tornquist et al. and Spencer et al., we saw a decrease in antigen specific response at day 0 between EPM and normal horses. However, in our study the *S. neurona* merozoite stimulated responses were increased in EPM vs control horses at day 1. The reason why day 1 samples in EPM horses had higher proliferation responses to *S. neurona* merozoites was unclear. It could be a higher concentration of merozoites in EPM day 1 samples, or the contamination of CV-1 cell debris in merozoites because some of the merozoites were not filtered before adding to cells. Since half of the EPM group consisted of shipped blood samples compared to blood samples from normal horses, the other possible explanation included that shipping and/or handling procedures probably changed lymphocyte proliferation responses to *S. neurona* merozites. As we saw in PWM stimulated proliferation responses, samples at day 1 had higher proliferation results than samples at day 0. Therefore, the results implied that there were some limitations in utilizing blood samples which has either been refrigerated or shipped overnight to a laboratory (day 1 samples) to assess cellular immune response,

particularly for CD8 and antigen specific immune responses. Additional studies are needed to investigate the validity and significance of these findings.

In immunophenotyping studies between EPM and normal horses, EPM horses had a higher percentage of CD4 cells in their peripheral blood. At this time, we could not determine whether the elevated CD4 cells in their peripheral blood was caused by *S. neurona* infection or other factors, such as a pre-existing level prior to infection. CD4+ T helper cells play an important role in optimal cell mediated immunity against intracellular pathogens, such as *T. gondii* (Tizard, 2002). Furthermore, Khan (Khan et al., 1996) showed that the percentage of CD4 T cell populations in the spleens from *T. gondii* infected mice increased by 7 days post infection. However, the CTLA-4 and CD28 markers were not expressed on those activated CD4 T cells and their DNA morphology was consistent with apoptosis. Our 7-AAD data showed that more than 90% of CD4 cells were viable. However, statistical analysis was not performed to conclusively state that there were no differences in EPM vs control horses. These data suggest that in EPM horses, the higher percentage of CD4 T cells may not undergo apoptosis. Additional studies are still needed to determine whether elevated CD4 cells in EPM horses are a consistent finding and the associated mechanisms.

Tornquist et al. reported (2001) that in the EPM horses employed in their study, EPM horses had a significantly lower percentage of CD4 cells in peripheral blood compared to neurologically normal *S. neurona* positive and seronegative horses. There were several possible reasons to explain the discrepancy between their results

and ours. The different results may have been caused by factors specific to the groups of EPM horses utilized (i.e. duration of infection and clinical signs), dose of *S. neurona* organisms, severity of disease, clinical stage (recovery from disease or progression of signs), or other factors. Another possible explanation for these differences was that our data may have been affected by the small sample size. The increased CD4 levels were statistically significant ($p < 0.05$) indicating that these differences were likely not due to chance. However, as the sample size (n=6 for day 0 samples) of the study was small and because the increased proportion of CD4 cells in PBMCs from EPM horses in day 0 were still within the normal range of CD4 level, it was possible that with a large group of EPM horses, we could not see these differences in CD4 populations. This significance did not appear in day 1 samples (n = 12), although there was a bias towards EPM horses having elevated CD4 levels vs normal horses. In addition, within the statistical analysis, each EPM horse was randomly matched with a normal horse to perform the analysis. If paired horses were not exactly consistent in age, breed, gender, and other aspects, it was difficult to conclusively decide that higher CD4 levels in EPM horses were caused by *S. neurona* infection. Therefore, further studies are still needed to confirm or disprove this finding and its associated significance.

Due to the limitations of this study, we were not able to match each EPM horse with a control horse of same age, breed, sex, and from the same farm. This had the potential to create some bias with the study. The other point was that the elevated CD4 levels were not present at day 1, although CD4 percentages were still increased in EPM horses. This could be due to an undetected effect of storage on CD4

populations. The 7-AAD data suggest that more than 90% of CD4 cells were viable at both day 0 and day 1 samples although statistical analysis was not performed to determine whether there was a significant effect of day. Based on our data from this study, additional studies including a larger sample size are needed to ascertain accuracy of CD4 differences as well as to assess CD4 function in EPM vs normal horses.

PMA/I-stimulated proliferation response was decreased in EPM horses compared to normal horses. To our knowledge, this study was the first report on the effects of the mitogen combination PMA/I on enriched lymphocyte proliferation in EPM horses. PMA stimulates cells to proliferate through binding to cell-surface receptor tyrosine kinases, which lead to activation of second messenger mitogen-induced protein kinase C (MAPK) and second messenger cAMP (Lodish et al., 2004). Once activated, MAP kinase translocates to the nucleus and activates transcription factors Activation Protein 1 (AP1), nuclear factor-κB (NF-κB1), and nuclear factor of activated T cells (NFAT) family. Members of the NFAT family of transcription factors pre-exist in the cytoplasm and are translocated to the nucleus to bind the promoter of genes needed for cell division and immune response (Thanos and Maniatis, 1995). Ionomycin acts primarily at the level of the internal calcium (Ca^{2+}) stores, increasing Ca^{2+} influx via activation of endogenous entry pathways (Liu and Hermann, 1978). Decreased proliferative responses to PMA/I in EPM horses may be due to changes in leukocyte subsets of PBMCs, cell apoptosis induced by PMA/I, or altered signialing in the PMA/I pathway associated with *S. neurona* infection.

Changes in leukocyte subsets of PBMCs could have been the most important explanation for the decreased proliferation response to PMA/I in EPM horses. We believed that ConA stimulated equine T lymphocytes, and PWM stimulated equine B lymphocytes. There were no significant differences in ConA and PWM stimulated responses between infected and non-infected horses, which meant that the proliferative capacity of T cells and B cells to these non-antigen specific mitogens was not affected by *S. neurona* infection. However, if PBMCs from EPM horses included higher percentages of other cell populations like monocytes, PMA induced proliferative responses could decline if PMA does not stimulate equine monocyte proliferation similarly to other cell populations (i.e. lymphocytes). However, Sugawara and Ishizaka (1983) demonstrated that PMA induced DNA synthesis in human monocytes, T cells, and B cells. Therefore, the question was whether there were some differences in monocyte activation and division among different species. To answer this point, additional studies can be designed to compare the proliferation rate to PMA between human monocytes and equine monocytes. Similarly, neutrophil contamination of samples could also affect proliferation. If the enriched leukocytes from EPM horses included more neutrophils than normal horses, the proliferation response to PMA/I stimulation could be reduced. However, our study on the effect of treatment by day and cytology data did not show any significant differences in the percentage of monocyte and neutrophils (Figure 3.5) between EPM and normal horses. These data suggested that contamination of monocytes and neutrophils in PBMCs was not the most likely explanation.

Another possible explanation for the differential PMA/I stimulated responses between EPM and normal horses was that there could be undetected differences in lymphocyte or leukocytes populations present (i.e. subpopulations). Differences in subsets may explain the lack of differences in ConA and PWM stimulated proliferation but suppressed *in vitro* PMA/I stimulated proliferation. There has been a PMA-responsive population identified in humans that has a high affinity to sheep erythrocytes, and this PMA/I population is distinct from that responding to ConA and phytohemagglutinin (PHA) (Touraine et al., 1977). If the PBMCs from *S. neurona* infected horses included decreased numbers of this PMA/I subpopulation than normal horses, the proliferation responses to PMA/I in infected horses could be decreased. Therefore, further studies are needed to investigate which subpopulations PMA/I stimulates in the PBMCs of horses. We could incubate each enriched subpopulation like CD4 cells, CD8 cells, B-cells, and monocytes separately with PMA/I to see the differences of the proliferation rates among each subpopulation. Alternatively, CFSE could be utilized to investigate which subpopulation of PBMCs does not respond to the stimulation of PMA/I.

Increased cellular apoptosis induced by PMA/I was one other possible reason to explain the decreased proliferation response to PMA/I in EPM horses. If PMA/I preferentially induced a different degree of cell apoptosis in immune cell subsets between normal and EPM horses, we would still observe a lower proliferation response to PMA/I in PBMCs from EPM horses. Unfortunately, our limited data on experimentally infected horses with EPM did not show statistically significant differences in PMA induced apoptosis between EPM and normal horses (see

Chapter 4 discussion for detail). Therefore, further experiments are needed, but at this time, this explanation was less likely.

Another alternative explanation involved different signaling pathways to stimulate cell proliferation by ConA and PWM, compared to PMA/I. Cyclosporin A (CyA) inhibited DNA synthesis induced by Con A, but did not inhibit the PMA-induced DNA synthesis (Sugawara and Ishizaka, 1983). PMA probably activated T cells via receptors that were different from those used by Con A. ConA, a plant lectin, bound very tightly to high-mannose-type N-glycans and very weakly to hybrid-type or biantennary complex-type N-glycans. Studies on Wistar rat thymocytes ruled out protein kinase C and Ca^{2+} as essential mediators of the proliferation responses associated with ConA and suggested that cAMP was involved in cell signaling (Grinstein et al., 1987). In contrast, PMA stimulated cells to proliferate through binding to cell-surface receptor tyrosine kinases, and activated cells to proliferate via protein kinase C and MAPK (mitogen-activated protein kinase) pathway (Calla and Wolfe, 1999). It was possible that *S. neurona* infection changed the receptor activity or signaling pathway specific to PMA stimulation. Further investigation will be discussed in the future studies.

Treatment Effects	CD4	CD8	B-cell	PMN	PMN/ DH59B+	Monocyte	Monocyte/ DH59B+
Day	0.2846	<0.001	0.2529	0.4205	0.1341	0.0867	0.192
Infection Status	0.155	0.5478	0.794	0.4709	0.6697	0.1487	0.5689
Day x Infection Status	0.2772	0.5098	0.2843	0.4569	0.95	0.9778	0.703

Table 3.1: *p*-value data for immune cell subsets comparing day 0 and day 1 samples. P-value data were assessed by the GLM procedure of the SAS system. Treatment effects include Day (day 0 and day 1) and Infection Status (uninfected and infected groups). The interaction between Day and Infection Status was the most interesting parameter because it reflected whether there was an effect of both Infection Status and Day. Eight randomly matched normal horses and EPM horses were included in this calculation. Significance was based on $p < 0.05$. CD4, CD8, and B-cell represent the percentage of each subset in the total lymphocyte populations. PMN (neutrophils) and monocyte represented the percentage of each subset in the total leukocytes. PMN/DH59B+ represented the percentage of DH59B positive PMNs in total PMNs. Monocyte/DH59B+ represented the percentage of monocytes that were DH59B positive of the total monocytes.

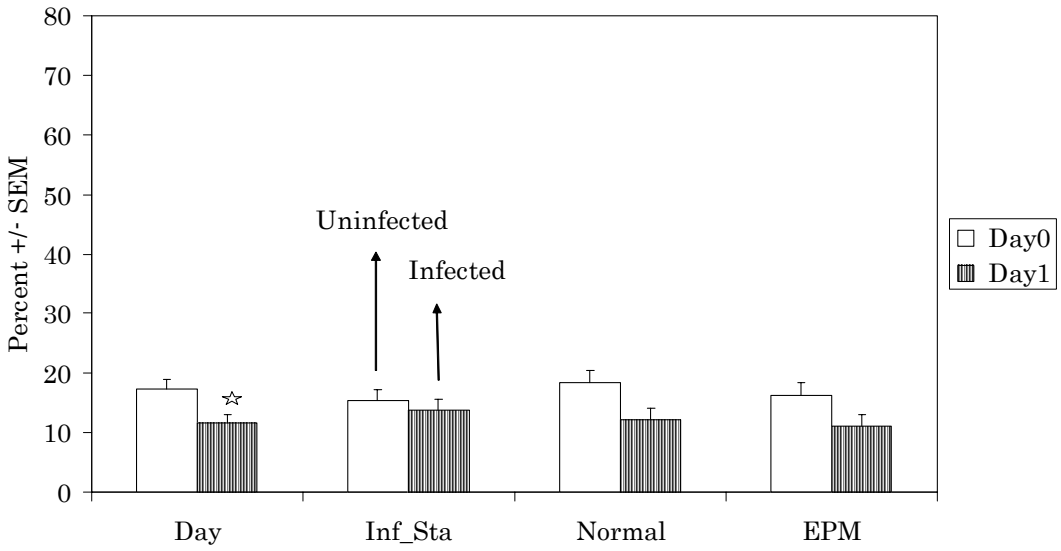

Figure 3.1: Percentage of CD8 T cells of total lymphocytes. Flow cytometry was performed to determine the percentage of CD8 lymphocytes after blood was immediately collected (day 0) and following overnight storage/shipment (day 1). Results were expressed as mean percentage ± SEM. N = 7 (7 normal and 7 EPM horses). Inf_Sta means Infection Status. Treatment effects include Day (day 0 and day 1) and Infection Status (uninfected and infected groups). Asterisk indicated a significant difference between day 0 and day 1 samples ($p < 0.05$).

Treatment Effects	Media	ConA (1µg/ml)	ConA (5µg/ml)	PWM (1µg/ml)	PMA/I (20ng/ml vs10pg/ml)	Live Merozoites (1x 10^5/ml)
Day	0.8388	0.5646	0.1461	0.0067	0.3384	0.0495
Infection Status	0.0996	0.4971	0.7688	0.4403	0.1164	0.7262
Day x Infection Status	0.4077	0.0783	0.0841	0.8629	0.5084	0.0092

Table 3.2: Statistical evaluation of proliferation results (*p*-value). Lymphocyte enriched leukocytes from EPM horses and controls were co-cultured with either non-antigen specific mitogens (ConA, PWM, and PMA/Ionomycin) or antigen specific mitogen (live *S. neurona* merozoites). Statistical analysis was performed based on delta ΔCPM's. Treatment effects include Day (day 0 and day 1) and Infection Status (uninfected and infected groups). The interaction between Day and Infection Status was the most interesting issue because it reflected the time point at which the significant differences appeared. *P*-value data were assessed by the GLM procedure of the SAS system. Nine randomly matched normal and EPM horses each were included in this analysis. Significant difference was assessed at $p < 0.05$.

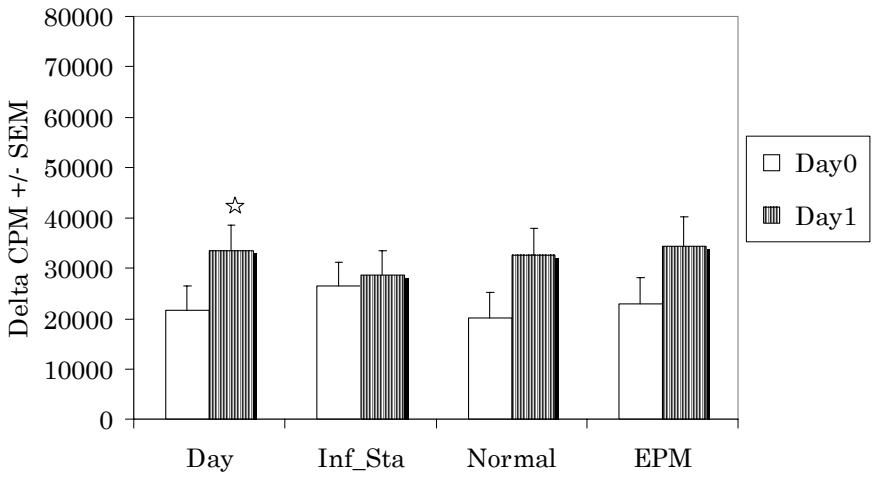

Figure 3.2: Pokeweed (PWM) stimulated proliferation assay comparing normal and EPM affected horses. Equine enriched leukocytes were stimulated with PWM (1 μg/ml)) for 48 hours followed by ^{3}H-thymidine for 18-24 hours. Proliferation response was measured by ^{3}H-thymidine incorporation. Delta counts per minute (ΔCPM) was calculated as the mean CPM of cells with a particular mitogen minus mean CPM of cells from unstimulated cells in media. Inf_Sta means Infection Status. Treatment effects included Day (day 0 and day 1) and Infection Status (uninfected and infected groups). Data for each group represented mean ± SEM of triplicates. For both day 0 and day 1, data from 9 normal and EPM horses each were used. Asterisk indicated a significant difference from day 0 to day 1 ($p < 0.05$).

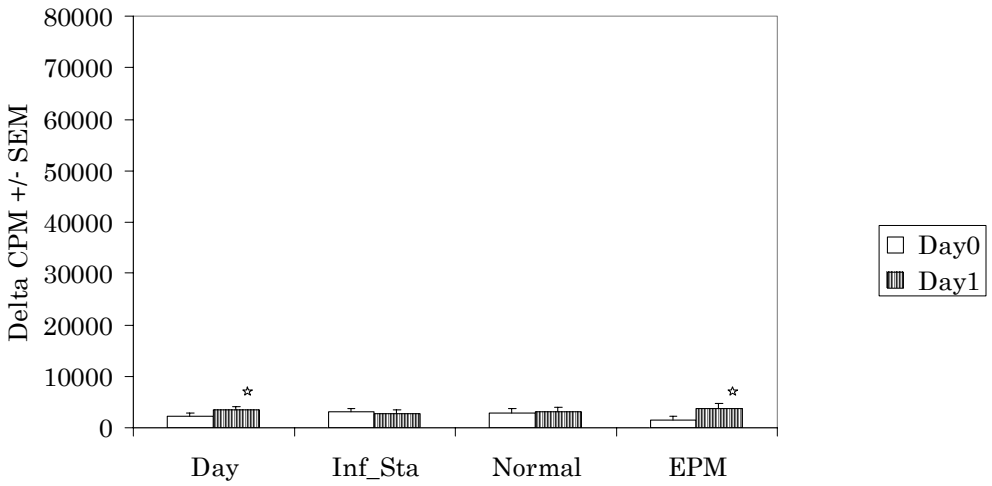

Figure 3.3: Blastogenesis assays stimulated by live *S. neurona* merozoites. Equine PBMCs were stimulated with live *S. neurona* merozoites for 48 hours followed by ^{3}H-thymidine for 18-24 hours. Proliferation responses were measured by ^{3}H-thymidine incorporation. Delta counts per minute (ΔCPM) was calculated as the mean CPM of cells with antigens minus mean CPM of cells from unstimulated cells in media. Inf_Sta means Infection Status. Treatment effects included Day (day 0 and day 1) and Infection Status (uninfected and infected groups). Data for each group represented mean ± SEM of triplicates. For both day 0 and day 1, data from 8 normal and EPM horses each were included. Asterisk indicated a significant difference from day 0 to day 1 ($p < 0.05$).

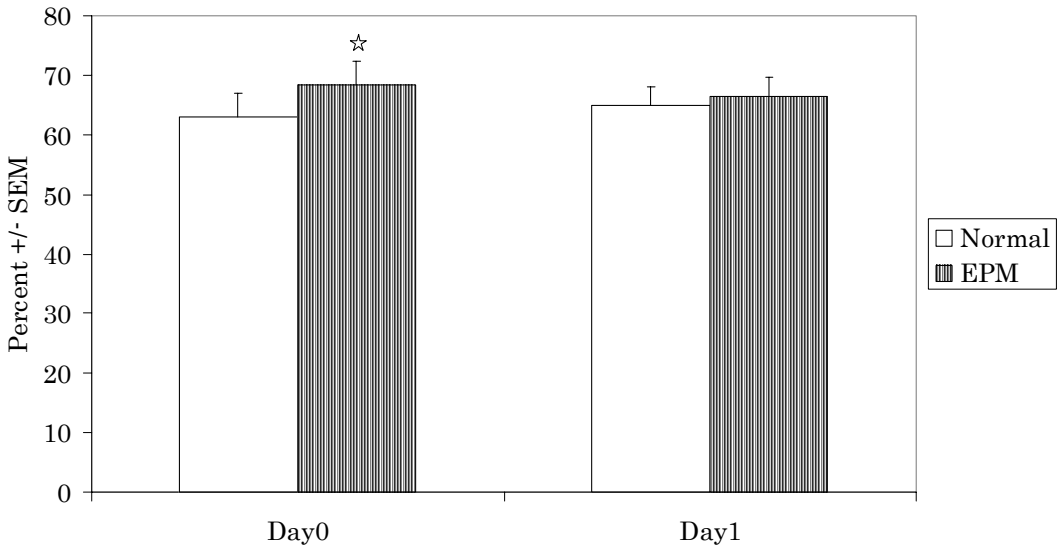

Figure 3.4: Percentage of CD4 cells of total lymphocytes between normal and EPM affected horses. Flow cytometry was performed to determine the percentage of CD4 cells as percentage of the lymphocytes. Results were expressed as mean ± SEM. For day 0, data from 6 normal and EPM horses each were used. For day 1, data from 12 normal and EPM horses each were included. Asterisk indicated a significant difference within day 0 samples ($p<0.05$).

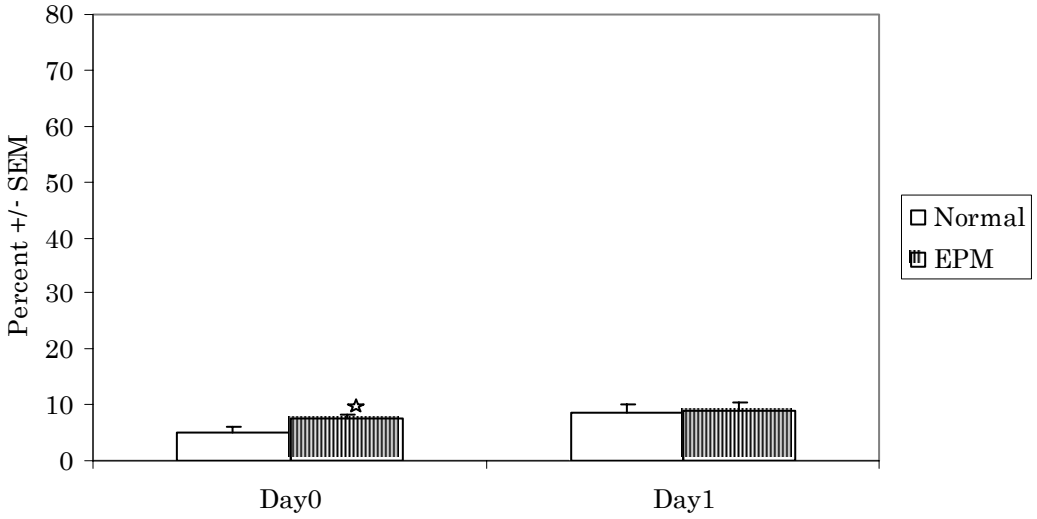

Figure 3.5: Percentage of neutrophils of total leukocytes. The percentage of neutrophils (PMN) was determined based on the flow cytometry data. Results were expressed as mean ± SEM. For day 0, data from 6 normal and EPM horses each were used and for day 1, 9 normal and EPM horses each were included in day 1 samples. Asterisk indicated a significant difference within day 0 samples ($p<0.05$).

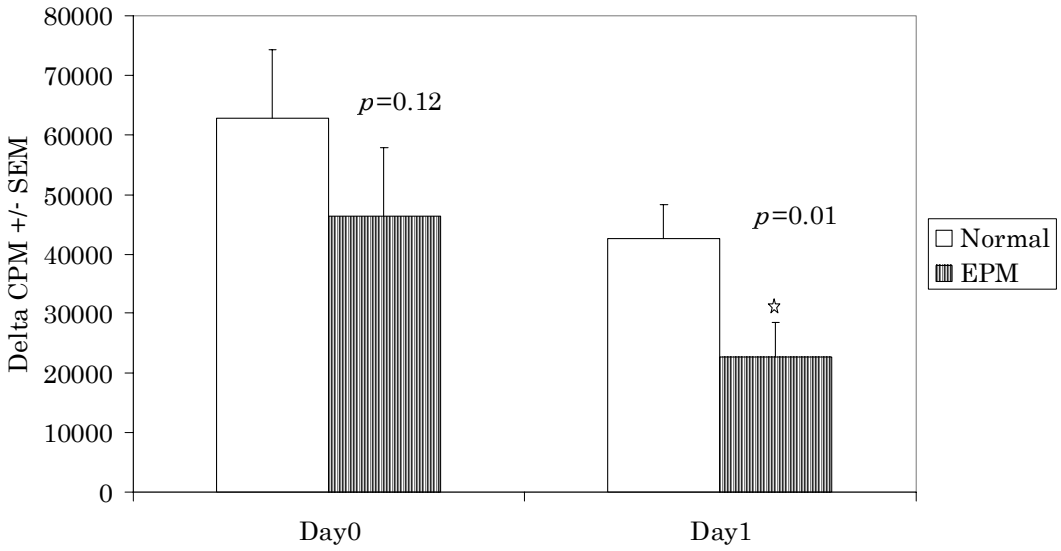

Figure 3.6: PMA/I stimulated proliferation assay comparing normal and EPM horses. PBMCs were stimulated with PMA/I for 48 hours followed by 18-24 hours of ^{3}H-thymidine incorporation. Delta counts per minute (ΔCPM) was calculated as the mean CPM of cells with mitogens minus mean CPM of cells from unstimulated cells in media. Results were expressed as mean ± SEM of triplicates. N is equal to 8 in day 0 samples and 15 in day 1 samples. Asterisk indicated a significant difference within day 0 samples (p<0.05).

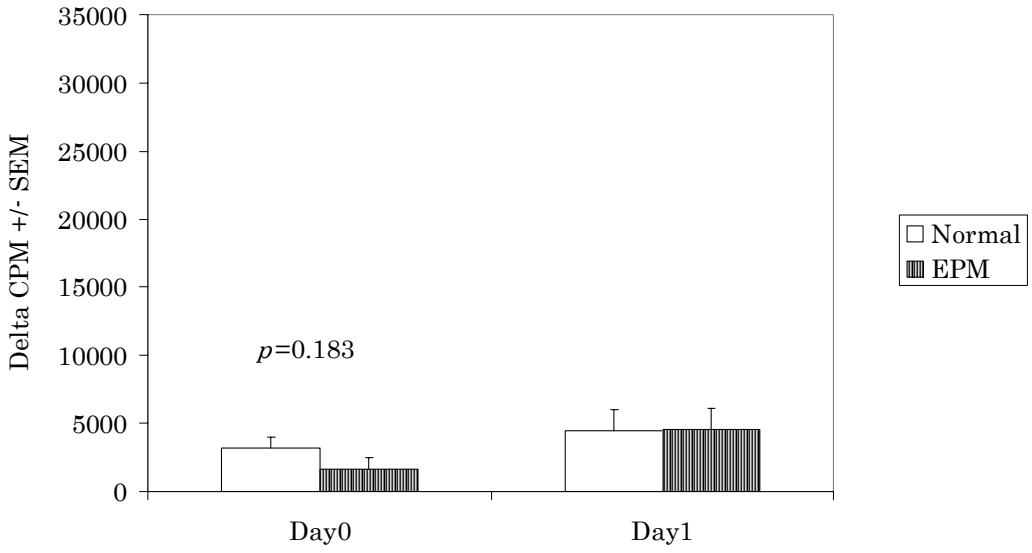

Figure 3.7: *S. neurona* merozoite stimulated proliferation assay comparing normal and EPM horses. PBMCs were stimulated with *S. neurona* merozoites for 48 hours followed by 18-24 hours of ^{3}H-thymidine incorporation. Delta counts per minute (ΔCPM) was calculated as the mean CPM of cells with mitogens minus mean CPM of cells from unstimulated cells in media. Results were expressed as mean ± SEM of triplicates. N is equal to 8 in day 0 samples and 15 in day 1 samples. Significance was assigned at $p<0.05$.

CHAPTER 4 Changes in immune subsets and function associated with experimental *S. neurona* infection of horses

4.1 Introduction

EPM has been one of the most commonly diagnosed neurologic diseases of horses in the US. The protozoan parasite, *S. neurona,* is the primary etiologic agent of EPM. Approximately 1% of horses in the United States developed signs of clinical infection, although a much higher percentage (40 – 80%) were seropositive depending on the location of the horses within the country (Saville, 1997). As the overall exposure was high, and only a small percent of the infected horses developed EPM, many investigators studying EPM hypothesized that immunocompromised horses were more likely to develop EPM (Dubey et al., 2001a).

As early attempts at developing an equine model were unsuccessful, mouse models were developed utilizing both C57BL/6 nude mice to study *S. neurona* mediated encephalitis (Marsh et al., 1997) and a γ-IFN knockout model to study drug efficacy and life cycle of *S. neurona* (Dubey and Lindsay, 1998). Employing these models, considerable research has been performed and subsequently knowledge gained regarding EPM.

In chapter 3, we measured the immune response to *S. neurona* in naturally infected and normal horses. Studies on naturally occurring cases helped us to determine the immune response status in horses that already had disease due to *S.*

neurona. However, as we did not know the immune status prior infection, this limited our interpretation of the results. Obviously, establishing an experimental horse model can overcome this weakness. Furthermore, utilizing a horse model would be ideal to enhance our understanding of the pathogenesis, develop improved diagnostics, develop more effective treatment therapies, and engineer an efficacious vaccine. Recently, after trying several different strategies, reproducible horse models have been generated (Fenger et al., 1997a; Cutler et al., 2001; Lindsay et al., 2000; Saville et al., 2001, 2004b; Sofaly et al., 2002). Among these, one successful model created by Ellison et al. (2004) involved immunocompetent horses that were infected with *S. neurona* infected homologous lymphocytes given intravenously. We collaborated with Dr. Ellison to perform this study whereby the horses were kept in Florida and samples were sent to Virginia Tech. Our goal was to determine the changes in immune function in response to *S. neurona* infection, as a means of understanding the pathophysiology of *S. neurona* infection in the development of EPM. We hypothesized that EPM horses had suppressed cell-mediated immune response compared to neurologically normal seropositive horses.

There have been limited information available on the immune dysfunction and pathophysiology of EPM in naturally occurring and experimentally infected cases of EPM. Tornquist et al. (2001) studied the lymphocyte proliferation responses (ConA 1ug/ml and *S. neurona* antigen 2.5ug/ml) and immunophenotypes (CD4, CD8, and monocytes) in naturally occurring EPM horses. They found a significantly lower proportion of CD4+ cells in symptomatic horses than that in both the seronegative and asymptomatic seropositive horses. Our data (See Figure 3.4, p56) was

inconsistent with the Tornquist et al. (2001) study. Tornquist and colleagues also found that PBMC (Peripheral Blood Mononuclear cells) from asymptomatic seropositive horses had higher proliferative responses to ConA and *S. neurona* antigen than PBMCs from both seronegative and symptomatic horses. These data suggested that asymptomatic horses have a strong immune response to *S. neurona* whereas both the antigen specific and non-specific T-cell immune responses were suppressed in clinically affected horses. Spencer et al. (2004) reported that lymphocytes from EPM horses were suppressed in both proliferative responses and IFN-γ production to SnSAG-1 compared to seronegative horses. Further studies by Spencer et al. (2005) demonstrated decreased IFN-γ production and increased expression of IL-4 in EPM vs normal horses. Our hypothesis was that EPM horses could have a suppressed Th1 and Tc1 immune response. Therefore, we employed Ellison's EPM model to determine how *S. neurona* infection altered the immune response over time. We assessed changes in immune cell subsets via flow cytometry, and immune function via blastogenesis assays and IFN-γ production to both antigen specific and non-specific mitogens.

4.2 Materials and Methods

4.2.1 Animals: Thirteen horses were included in this study. Eleven of thirteen (11/13) horses were yearlings. There was one horse each that was 12 and 19 years old. Horse breeds included 1 Arabian, 1 Paint, and 11 Quarter horse or Quarter horse crosses. There were 4 fillies, 3 geldings, and 6 stallions. The 13 horses were kept on pasture with free exercise at one farm in Florida. Horses were vaccinated, dewormed,

and had Coggins tests performed. Cervical films were normal on all horses. Baseline neurologic examinations were performed. Baseline serum and CSF were collected for analysis by WB and SAG-1 ELISA. All horses were negative for *S. neurona* specific antibodies in the CSF by WB and SAG-1. All horses were negative for *S. neurona* serum antibodies by SAG-1 and 2/13 were low positive by WB. Blood samples were collected at day minus 13 for baseline data. Horses were randomly assigned such that there were 5 control horses and 8 infected horses, the latter of which would be challenged daily for 14 days with 6000 *S. neurona* merozoites per day (*S. neurona* path 3) administered via infected autologous leukocytes. Beginning at day 14, horses were lounged daily 5 min each direction. Additional blood samples were collected at the following time points: day 1, day 2, day 5, day 13, day 28, and day 48 post infection. At each time point, blood from those horses was shipped to us at approximately 4^0C. At the same time, blood was collected from another 2 female horses from Veterinary Teaching Herd to be used as internal controls to monitor how the shipping condition would affect results. The internal controls include a 7 year-old Thoroughbred, and a 20 year-old Appaloosa mare. Blood from internal control horses was kept in the refrigerator overnight after a blood smear was made to perform a differential to assess the effects of storage. Horses had neurologic examinations, CSF taps, serum and CSF analysis by SAG-1 ELISA and WB at days 28 and 55 PI. All infected horses were serum and CSF positive for *S. neurona* specific antibodies by the end of the study. Seven of 8 infected horses had grade 2 neurologic score by the end of the study. One horse was only grade 1 affected. Animal care protocols were approved by the Institutional Animal Care and Use Committee at Virginia Tech.

4.2.2 Isolation of Peripheral Blood Mononuclear Cells (PBMCs): PBMCs were isolated by density gradient centrifugation (Witonsky et al., 2003c). Whole blood at room temperature was diluted with Phosphate Buffered Saline (PBS, Mediatech, Herndon, VA) at the ratio of 1:2 before carefully layering the diluted blood (2:1) on Lymphoprep (Lymphoprep 1.077, Greine, NJ). In our experience, all these reagents must be at room temperature for optimal purity of lymphocytes, otherwise there can be considerable neutrophil contamination. Samples were centrifuged at 1,500 rpm (350 x g) for 30 min at 23 ^{0}C without the brake. The buffy coat was collected and diluted with minimally an equal volume of PBS. Cells were centrifuged at 1,300 rpm (250 x g) for 10 minutes at 4 ^{0}C. The supernatant was removed and samples were resuspended in PBS, and washed twice, spinning at 1,300 rpm (250 x g) for 5 minutes at 4 ^{0}C. Cell counts were determined with the CASY-1 (TTC cell counter and analyzer system, Sharfe System, GMbH, Reutingen, Germany). Cells were resuspended to a final concentration of 2 x 10^6 / ml in complete RPMI 1640 (Cellgro^{TM} RPMI 1640 1x, Thomas Scientific, Herndon, VA) with L-glutamine, Hepes Buffer 25 mM, 10% heat inactivated fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), penicillin (50 IU/ml), and streptomycin (50 IU/ml) (Sigma Chemical Co, St Louis, MO).

4.2.3 Cytospin preparation: Cells (0.25 x 10^6 cells) were diluted with 100 µl PBS and loaded to a centrifugation chamber. Each chamber was placed into a cytocentrifuge (Cyto-Teck centrifuge, Sakura Finetechnical Co, Tokyo, Japan), and spun at 500 rpm (30 x g) for 5 minutes. The slides were air-dried followed by fixing

and staining with modified Wright stain. Cover slips were placed, and 100 cells were enumerated and described as the percentages of lymphocytes, neutrophils, macrophages, basophils, or eosinophils (Witonsky et al., 2003c).

4.2.4 Flow cytometry: For each sample, 5 x 10^5 cells were placed in a 96 well round bottom plate (Corning Glass Works, Corning, NY). Cells were washed with 4 ^{0}C PBS and centrifuged at 1,300 rpm (250 x g) for 5 min at 4 ^{0}C. The following monoclonal antibodies were added to individual samples: CD4 (mouse anti-equine CD4 antibody, cell line HB61A, IgG_1, VMRD, Pullman, WA), CD8 (mouse anti-equine CD8 antibody, cell line HT14A, IgG_1 VMRD, Pullman, WA), B-cell (mouse anti-equine CD5 antibody, cell line B29A, IgG_{2a}, VMRD, Pullman, WA), and DH59b antibody (mouse anti-equine IgG_1, VMRD, Pullman, WA) to equine granulocytes. Cells were incubated with 0.5 μg of the primary antibody for 20 min at 4 ^{0}C. Cells were washed, and then incubated for 20 min at 4 ^{0}C with 0.5 μg secondary antibody of either FITC rat anti-mouse IgG_1 (for conjugation to CD4, CD8, and DH59b antibody) (Pharmingen, San Diego, CA) or PE rat anti-mouse IgG_{2a} (for conjugation to B-cell antibody) (Pharmingen, San Diego, CA) at a concentration of 1 μg/10^6 cells. Unstained samples were incubated with PBS. Cells were washed and resuspended in 200 μl PBS (4 ^{0}C) for flow cytometry (EPICS XL flow cytometer, Coulter, Hialeah, FL). 7-AAD (as described below) was added prior to running the samples on the flow cytometer.

4.2.5 7-amino actinomycin D (7-AAD) staining: After the samples were stained with the primary and second antibodies, followed by washing and resuspending the

samples in PBS, 1 μg of 7-AAD (Molecular Probes, Eugene, OR) was added to each sample. The plate was incubated for no more than 30 min on ice in the dark. Five or ten thousand cells gated events per sample were collected by flow cytometry (Schmid et al., 1992). Based on the intensity of staining, cells were classified by their subset and as $7AAD^{dull}$ (live cells), $7AAD^{moderate}$ (early apoptosis), and $7AAD^{bright}$ (late apoptosis) (Gogal et al., 2000).

4.2.6 Live merozoite preparation: *S. neurona* merozoites (SN-37R) (Sofaly et al., 2002) were grown and maintained in African green monkey (*Cercopithecus aethiops*) kidney cells (CV-1 cells, ATTC CCL-70, American Type Culture Collection, Rockville, MD, USA). *S. neurona* merozoites were harvested from CV-1 cells by removing the complete media (RPMI with L-glutamine, hepes buffer 25 mM, 2% heat inactivated FBS, 50 IU/ml of penicillin/streptomycin solution, 1% sodium pyruvate solution, Mediatech, Herndon, VA). The suspension was filtered through a 3 μM filter (filter) and spun at 1,500 rpm (350 x g) for 10 min at room temperature, and then resuspended in complete media. The merozoites were enumerated with a hemacytometer and resuspended at a concentration of 1×10^5 /ml with complete media containing 10% heat inactivated FBS (Lindsay et al., 2000).

4.2.7 Lymphocyte Proliferation Assays: A 100 ul aliquot of equine enriched lymphocytes (2×10^6/ml) was cultured in triplicate wells together with 100 ul of the appropriate mitogen or live merozoites (1×10^5/ml) (Witonsky et al., 2003c). Final concentrations of mitogens in the wells were 1 μg/ml or 5 μg/ml ConA (Sigma Chemical Co, St Louis, MO), 1 μg/ml PWM (Sigma Chemical Co, St Louis, MO),

20 ng/ml PMA and 10 pg/ml ionomycin (Sigma Chemical Co, St Louis, MO). Cells were incubated at 37 ^{0}C in humidified 5% CO_2 and pulsed with 1 μCi ^{3}H-thymidine after 48 hour culture. Plates were harvested 18-24 hours later using a Filtermake Harvester (Packard Bioscience, Billerica, MA). Delta counts per minute (ΔCPM) were calculated based on the mean CPM of cells with mitogens minus mean CPM of cells from unstimulated cells in media (spontaneous proliferation).

4.2.8 SnSAG1 plate incubation: 96-well round bottom plates were coated with 50 μl SnSAG1 (10 μg/ml) in PBS and incubated overnight at 4 ^{0}C. The plate was washed with PBS three times and 100 μl enriched lymphocytes (2 x 10^6/ml) 100 μl of complete media were added. Wells with cells only without SnSAG1 coating were used as negative controls. Cells stimulated by ConA (5 μg/ml) were positive controls. The plate was incubated 72 hours before adding ^{3}H-thymidine. After that, cells were incubated for another 18 to 24 hours and then harvested with Filtermake Harvester as described above.

4.2.9 IFN-γ intracellular cytokine staining: A modified protocol (Belz et al., 2001; Hines et al., 2003) was used. Briefly, cells (5x10^5/well) were incubated in complete media (RPMI 1640 with L-glutamine, Hepes Buffer 25 mM, 10% heat inactivated fetal bovine serum, 50 IU/ml penicillin, and 50 IU/ml streptomycin) with Brefeldin (1μg/ml) (Epicentre, Madison, WI) overnight and 100 μl one of the following stimulants: ConA (10 μg/ml), live merozoites (1x10^4/well), or media only. After overnight incubation, cells were centrifuged down at 1,300 rpm (250 x g) for 5 min at 4 ^{0}C and then washed in flow buffer (1% BSA, Sigma, St. Louis, MO) contaning

5 μg/ml Brefeldin A. Cells were stained with 50 μl of the primary cell surface antibody (1:100 dilution in flow buffer containing 5 μg/ml Brefeldin A) either CD4 (mouse anti-equine CD4 antibody, cell line HB61A, IgG1, VMRD, Pullman, WA) or CD8 (mouse anti-equine CD8 antibody, cell line HT14A, IgG1 VMRD, Pullman, WA) (1μg/1 x 10^6 cells) for 20 min at 4 ^{0}C. Cells were washed with 100 μl PBS containing 5 μg/ml Brefeldin A, and then incubated with a secondary antibody, rat anti-mouse FITC-IgG1 (Pharmingen, San Diego, CA) (Witonsky et al., 2003c) for another 20 min at 4 ^{0}C. Cells were washed and fixed in 100 μl of 2% paraformaldehyde (Fisher, Pittsburgh, PA) plus 100 μl of PBS for 15 min at 4 ^{0}C. Cells were washed twice and then permeabilized with 0.5% saponin (Sigma, St. Louis, MO) for 10 min at room temperature. Bovine-IFN-γ antibody (Mouse anti-bovine IgG1, SeroTec, Raleigh, NC) was complexed with Zenon-PE-IgG1 beads (Molecular Probes, Carlsbad, California) following product instructions. The complex was added to the cells and incubated for 30 min at 4 ^{0}C. Following incubation, cells were washed, and 5,000 gated events per sample were analyzed by the Coulter flow cytometer.

4.2.10 PMA/I induced lymphocyte apoptosis: This experiment was designed to determine whether PMA/I can differentially induce apoptosis in PBMCs from EPM vs control horses. The degree of apoptosis was measured by 7-AAD staining. At the following time points: day 2, day 5, day 13, and day 48, PBMCs were isolated from shipped blood as described previously. Cells (5 x 10^5/well) were resuspended in complete media and incubated with PMA/Ionomycin (final concentration at 20 ng/ml and 10 pg/ml) or media only overnight at 37^{0}C, 5% CO_2. Cells were washed

and then aliquots of cells were stained with CD4, CD8, B29A, or DH59b antibodies followed by secondary antibody FITC-IgG_1 or PE-IgG_{2a} (Witonsky et al., 2003c). After the second wash, 1 µg 7-AAD (Molecular Probes, Eugene, OR) was added to each sample. The plate was incubated for no more than 30 min on ice in the dark. Five thousand gated events per sample were analyzed via flow cytometry.

4.3 Statistical Analysis

Analysis of variance was conducted using the GLM procedure of the SAS system (version 8.2, SAS Institute Inc., Cary, NC) to test for treatment effects (Date and Infection Status) and covariate (neutrophil). Standard residual plots were used to assess model adequacy. Tukey-Kramer t test was used to establish differences. Significant interactions were further investigated by testing for simple main effects using the the SLICE option. Results were expressed as mean ± SEM.

4.4 Results

*4.4.1 Effects of S. neurona infection on immunophenotypes of experimentally infected horses***:** Changes in immune cell subsets due to *S. neurona* infection in the horses were analyzed by flow cytometry. 7-AAD data to detect cell viability in experimentally infected horses were similar in CD4 and CD8 cells to that in naturally infected horses. The percentages of viable cells in CD4 and CD8 were above 90%, and there were no statistical differences in CD4 viable cells, CD4 cells in early apoptosis, CD8 viable cells, and CD8 cells in early apoptosis between

infected and uninfected horses. Typically, the percentage of viable B-cells ranged from 60% to 90%, however, viability for some B-cell samples was between 20-40%. At this time, a correlation with the low viability was not evident. Howeve, we suspect that temperature may significantly affect B-cell viability. There were no statistical differences in viable B-cells and B-cells in early apoptosis between infected and uninfected horses, but at day 48 PI, infected horses had significantly lower percentage of viable B-cells compared to uninfected horses (p = 0.0144). Cytology data showed a strong relationship between blood temperature and neutrophil contamination in PBMCs, which was consistent with the data from naturally infected horses. There were no statistically significant differences in CD4 cell, CD8 cell, B-cell, monocyte or neutrophil (PMN) populations at any of the different time points between unchallenged horses and challenged horses. The percentages of CD4 in PBMCs were presented in Figure 4.1. There were no significant changes associated with any of the time points with *S. neurona* infection. Figure 4.2 illustrated the data on CD8 populations. Baseline data from the population that would be infected had a significantly lower percentage of CD8 (11.54% ± 1.16) in PBMCs at 13 days before infection compared to uninfected treatment group (15.04% ± 1.39). No significant differences ($p > 0.05$) were present between these two groups of horses at any other time points post infection. Figure 4.3 depicted the differences in the percentage of B-cells between infected and non-infected horses. Values at day 28 post-infection were significantly different from data on other time points. However, there were no significant differences between infected and non-infected horses at any of the time points. Further, there were no significant differences in the percentages of monocytes and neutrophils in PBMCs

between challenged horses and unchallenged horses at any time points (data not shown).

4.4.2 Analysis of Lymphocyte Proliferation Assays: Results from *in vitro* mononuclear cell proliferation in response to mitogens (ConA, PWM, PMA/I, and live merozoites) were summarized in Table 1. The covariate neutrophil significantly ($p < 0.0001$) affected the PMA/I stimulated proliferation response. There were no significant differences in the proliferation assays stimulated by ConA, PWM, and live merozoites (Figure 4.8a). However, we did see that the experimentally infected horses had lower proliferation responses to PMA/I (Figure 4.4) than control horses, which was consistent with results in naturally infected EPM horses. There were not significant differences in SAG-1 (Figure 4.8b) stimulated proliferation between infected and normal horses ($p = 0.4458$). Neutrophil contamination significantly affected SAG-1 stimulated proliferation responses ($p = 0.0021$).

4.4.3 Analysis of IFN-γ intracellular cytokine staining: There were no significant differences between IFN-γ production by CD4 or CD8 cells stimulated with ConA or *S. neurona* merozoites from *S. neurona* infected or uninfected horses. Table 2 showed the results of the statistical analysis based on p-values for IFN-γ intracellular cytokine production. A difference in the percentage of CD4 or CD8 cells that produced IFN-γ between challenged and non-challenged horses was not detected. Figure 4.5 presented: a) the percentage of CD4 cells producing IFN-γ of total CD4 cells following ConA stimulation, and b) the percentage of CD4 cells of total lymphocytes producing IFN-γ following ConA stimulation. *S. neurona* merozoite-

stimulated CD4 cells from the challenged and control horses showed similar results for IFN-γ production (data not shown). Figure 4.7a and b presented the percentage of CD8 cells following overnight stimulation with ConA ($p < 0.05$) and *S. neurona* merozoites ($p = 0.08$). Figure 4.7c and d presented the percentage of CD8 producing IFN-γ following overnight stimulation with ConA ($p = 0.10$) and *S. neurona* merozoites ($p = 0.06$), respectively.

4.4.4 Effects of PMA/I on lymphocyte apoptosis: The results of the p-values from the analysis were shown in Table 4.3. This table only included the responses with significant differences. There were no statistical differences in the degree of cellular apoptosis in CD4 cells and B-cells. As one can see, the percentage of CD8 in total lymphocytes were significantly different due to the infection status, not necessarily associated with PMA/I. There was a trend ($p = 0.1952$) that PMA/I caused some differences in the percentage of CD8 cells experiencing early apoptosis between infected and non-infected horses (Figure 4.6). At day 13 PI, uninfected horses had higher percentage of CD8 cells in early apoptosis compared to infected horses. For the PMNs incubated with PMA/I, similar results as those presented for CD8 cells were apparent at day 13 post infection. Other notable results were that there were significant differences in the percentage of PMNs in early apoptosis with and without PMA/I.

4.5 Discussion

Cell-mediated immune responses are essential for host control of intracellular infections (Tizard, 2000). *S. neurona* is a protozoan parasite that infects multiple vertebrate species, and horses are abnormal intermediate hosts (Fenger et al., 1997a). Extensive studies on a similar protozoan, *Toxoplasma gondii*, observed that treatment with monoclonal IFN-γ antibody in mice eliminates resistance to acute toxoplasma infection and results in chronic toxoplasmosis (Suzuki et al., 1988; Suzuki et al., 1989; Gazzinelli et al., 1991). Treatment with anti-IFN-γ antibody also induced neurologic signs in *S. neurona* infected SCID mice that had natural killer (NK) cells depleted (Sellon et al., 2004a). Furthermore, *S. neurona* infected IFN-γ KO mice developed encephalitis (Dubey and Lindsay, 1998). Considering the similar characteristics between these two parasites and based on published data (Dubey and Lindsay, 1998; Spencer et al., 2004, 2005; Witonsky et al., 2003 b, 2005 a), we proposed that IFN-γ plays a major role in resistance in horses against *S. neurona*.

Currently, limited information exists about the immunopathogenesis of EPM. This study was the first of its kind in experimentally infected horses with *S. neurona* to focus on the changes in the immune response, including IFN-γ production. In this study, we did not observe significant differences in IFN-γ production associated with *S. neurona* infection. This may be, in part, due to the result of the experimental method we employed in this study, such as sub-optimal merozoite and SnSAG-1 concentrations and conditions. Intracellular IFN-γ staining only detected the percentage of IFN-γ positive CD4 or CD8 cells and the total number of IFN-γ positive CD4 or CD8 cells, but cannot quantify the total amount of IFN-γ produced

by each cell. Results from our studies showed that about 80% of CD4 and CD8 cells were IFN-γ positive CD4 or CD8 cells in response to ConA (Figure 4.5a). Unfortunately, these results could not give any information about the differences in the amount of IFN-γ produced by the individual CD4 or CD8 cell between EPM and normal horses. However, in most situations, when there is an upregulation in expression or production by a cell, there is a shift in fluorescence intensity. This can be detected by flow cytometry. We described these populations as high vs. low or bright vs. dull. We did not see a shift in the intensity of the population, which suggested that it was possible, but less likely, that there was any difference in IFN-γ production by individual CD4 or CD8 cells from EPM affected horses. Additionally, we did not measure IFN-γ production by other cell types (i.e. monocytes). Total IFN-γ could be measured by ELISA. However, to date, an equine specific assay is not commercially available. Spencer et al. (2004) found that the EPM horses did not produce IFN-γ as detected by RT-PCR (Reverse transcriptase-polymerase chain reaction) after 72 hr stimulation with SnSAG-1 while EPM-negative horses produced IFN-γ after 24 hr co-culture with SnSAG-1. Further studies by Spencer et al. demonstrated a decrease in IFN-γ production and increase in IL-4 production (Spencer et al., 2005). Spencer's studies included both day 0 and day 1 samples, but the cell viability of samples at day 1 was not measured (personal communication with Dr. Spencer). On the other hand, there were some significant differences among different time points in our study, which suggested that shipping condition could change the percentage of CD4/CD8 cells producing IFN-γ. This implied that careful control of shipping conditions was necessary in order to properly evaluate shipped samples, and there may be some limitations in using shipped samples.

Although there were no significant differences in overall *p*-values (Table 4.2), there were some trends in differences in IFN-γ production by CD8 cells between control and EPM affected horses. There was a trend, at day 5 PI, that the percentage of CD8 cells producing IFN-γ ($p = 0.10$) decreased following the overnight incubation with ConA (Figure 4.7c), and increased at day 13 following the overnight incubation with *S. neurona* merozoites (Figure 4.7d) compared to uninfected horses. This suggested that there may be an early (day 5 PI) suppression in CD8 Tc1 immune response following experimental infection of *S. neurona*.

Since Spencer and colleagues did not measure the cell viability for their samples, it could be possible that the decreased IFN-γ and increased IL-4 production might be due to lower percentage of viable lymphocytes. Alternatively, lymphocyte purity was unknown in their study. As our study showed, the neutrophil contamination could have affected the results of immune function tests. Finally, the results would have been more creditable if their experiments included negative controls (i.e. lymphocytes incubated with PBS, not SAG-1), positive controls (i.e. ConA or another non-specific mitogen) and more time points. Additionally, the results of the negative RT (negative control) and positive control (B-actin) were not given. These results were important, as they used 21 horses in each group. However, at each time point, there were only a limited number of samples (i.e. <50%), that expressed at least one cytokine gene. Without having the results of negative and positive controls, it limits the credibility of the data. It was possible that all of these samples were analyzed, but none of the results were given. Additionally, at the 48 hr time point, 10 of 21 seronegative horses were IFN-γ positive, and none of the 21 EPM horses

produced IFN-γ. But at 72 hr time point, none of the seronegative horses produced IFN-γ, and 4 of 21 EPM horses were IFN-γ positive. This at least suggested that the time points to measure IFN-γ production were critical. It would have been helpful, if data from a 24 hr time point were collected. Overall, based on the lack of information provided with the data, caution is needed in interpreting the significance of the data from the study by Spencer et al. (2005).

In experimentally infected horses, the proliferation response to PMA/I decreased significantly compared with non-infected horses on days 2 and days 28 PI. This result was in agreement with our previous results from naturally infected horses. To our knowledge, this was the first report that PMA/I–stimulated PBMCs from experimentally infected EPM horses demonstrated suppressed proliferation responses compared to normal horses. The possible reasons to explain reduced proliferation responses to PMA/I have been discussed in detail in Chapter 3. Briefly, the decreased response could be due to changes in leukocytes subsets of PBMCs, cell apoptosis induced by PMA/I, or altered signaling in the PMA/I pathway associated with *S. neurona* infection. As previously discussed regarding apoptosis induced by PMA/I, based on the limited data that we had, we did not observe significant differences in the degree of cell apoptosis associated with PMA/I overnight incubation. Since there was no significant difference in ConA and PWM responses between infected and non-infected horses, the proliferative capacity of T cells and B cells to other non-antigen specific mitogens was not affected by *S. neurona* infection. However, if PBMCs from EPM horses included higher percentage other cell populations, such as monocytes or neutrophils, PMA induced

proliferative responses may decline. However, cytology data did not show any differences in the percentage of monocytes and PMN in the total of PBMCs between experimentally infected horses and non-infected horses. Alternatively, there could be differences in the lymphocyte populations (i.e. subpopulations) stimulated by PMA/I from that by ConA and PWM. As previously stated, differentially responsive populations have been described in humans (Touraine et al., 1977). If the PBMCs from *S. neurona* infected horses included reduced numbers of this PMA/I subpopulation compared to that in normal horses, the proliferation response to PMA/I in infected horses will decrease. To date, we have not assessed activation status (i.e. naïve, effector, memory) or Th/Tc status. It is also possible that different subpopulations of CD4 or CD8 cells could differentiallly respond to PMA/I. Therefore, further studies are needed to investigate the differentially responsive PMA/I stimulated subpopulation in horses. We could incubate each enriched subpopulation such as CD4 cells, CD8 cells, B-cell, and monocytes separately with PMA/I or PMA alone to detect the differences in proliferation rates among each subpopulation. Alternatively, differential proliferation could be assessed based on CFSE incorporation as detected by flow cytometry. Regarding the alterations in cells associated with PMA/I from EPM and normal horses, we suggest that *S. neurona* could "directly" interfere with binding of PMA/I to the cell surface by sharing the same cell surface glycoprotein or altering the glycoprotein structure when infecting the cells. However, a more likely explanation is that there could be an indirect effect of *S. neurona* on PMA/I induced signaling. In this case, inflammation from infected cells or direct infection of the cells somehow alters PMA/I induced signaling. To determine if there is an effect, we may test the activity or concentration of tyrosine

kinases and signaling molecules, i.e. Raf, MAPK/ERK kinase (MEK), extracellular signal-regulated protein kinase (ERK), in the PMA signaling pathway, protein kinase C isoenzymes, and MAPK in infected cells.

Alternatively, Ellison et al. (2001) indicated that calcium ionophore could release intracellular *S. neurona* merozoites from different cell lines at a concentration of 1 μM when incubated at 37^0C for 40 min. If the PBMCs incubated with PMA/I from infected horses contained *S. neurona* merozoite infected cells, merozoites could be released causing inflammation which would inhibit cell proliferation and/or merozoites could infect other PBMCs. This could possibly result in the lower proliferation response to PMA/I in EPM horses compared to normal horses. However, there are two points to elucidate before we propose it as a likely explanation. One is that the concentration of ionomycin (10 pg/ml) in this study is 7.43×10^{-3} dilution compared to that used by Ellison. Does this diluted ionomycin still release merozoites from host cell lines? Another question is whether PBMCs from EPM horses include *S. neurona* merozoites in their cytoplasm? Therefore, additional experiments are needed to explore this possibility.

Mitogens, like ConA, have been employed by other investigators to understand whether lymphocytes from EPM horses had decreased non-specific stimulated proliferation responses. Tornquist (2001) studied the lymphocyte proliferation responses (ConA 1ug/ml and *S. neurona* antigen 2.5ug/ml) on naturally occurring EPM horses, and found a higher PBMC proliferative response to ConA and *S. neurona* antigen in asymptomatic seropositive horses than that in both the

seronegative and symptomatic groups, which suggested that asymptomatic horses have a strong immune response to *S. neurona* whereas the antigen specific immune response was suppressed in clinical EPM cases. However, Spencer et al. (2004) reported that there were no significant differences between EPM-positive and -negative horses in lymphocyte proliferation responses to ConA. Our current proliferation results with ConA was consistent with Spencer et al. (2004). These unreconciled differences were possibly due to differences in EPM affected horses with respect to duration of disease, dose of *S. neurona*, and severity of disease.

Based on the limited data (Table 4.3), the amount of apoptosis in PMN populations between challenged and unchallenged horses was significantly different for cells stimulated with and without PMA/I. It is difficult to determine whether this difference was due to PMA because, as reported, PMA is a tumor activator and can inhibit cell apoptosis at both the transcriptional and post-transcriptional level (Herrant et al., 2002). PMA activates the Raf/MEK/ERK pathway efficiently via PKC activation in many cell types, including Jurkat cells (Li et al., 1999). Furthermore, Takagi (Takagi et al., 2004) reported that PMA could activate extracellular signal-regulated kinase (ERK) which provides protection for Jurkat cells against methylglyoxal-induced apoptosis. In our results, significant differences in early apoptosis only appeared in PMN with or without the stimulation of PMA/I. However, there was a trend in day 13 PI that the non-infected group of horses had a higher percentage of CD8 cells experiencing apoptosis than CD8 cells from infected horses (Fig 4.6). For monocytes and neutrophils, the same significant differences also appeared at day 13. One explanation for the difference in apoptosis was that

uninfected horses had lower percentage of CD8 cells (11.7451 ± 1.6947%) of total lymphocytes than infected horses (19.7087 ± 1.0202%), which could cause a relatively higher percentage of CD8 cells in early apoptosis in uninfected horses. Another possibility is that CD8 cells from infected horses were responding to antigenic stimulation (activated CD8 cells), which decreased their sensitivity to apoptosis. Unfortunately, we did not collect samples at day 28 in which we saw significantly lower proliferation responses to PMA/I stimulation in EPM horses. Samples from day 28 could provide more information about the PMA/I induced cellular apoptosis.

The immunophenotyping study in experimentally infected EPM horses did not detect significant differences in CD4, CD8, B-cells, monocytes, and/or neutrophils in peripheral blood (day 1). This was consistent with our previous study on naturally infected horses (Figure 4.4, Chapter 3) for day 1 samples. However, we did observe a higher percentage of CD4 cells in peripheral blood from EPM horses at day 0. Studies by Tornquist et al. (2001) found a significantly lower proportion of CD4+ cells in symptomatic horses than in both the seronegative and asymptomatic seropositive ones. The different results could have been caused by differences in the investigated populations of horses employed for the studies with respect to time since infection, dose of organism, severity of disease, response to disease and/or treatment (recovery, deterioration), or some other related conditions. This implies that there are several factors which have potential to introduce variability in studies on immunopathogenesis of EPM in horses, such as breed, age, gender, farm environment condition, virulence of *S. neurona* strain. We expect that the largest

variable involves *S. neurona* (strain, dose, duration of disease, etc). However, these factors have the potential to cause variability within the results. These variables may contribute to the differences seen in CD4 levels in EPM affected horses. Further studies are still needed to determine the effects of *S. neurona* infection on CD4 cells.

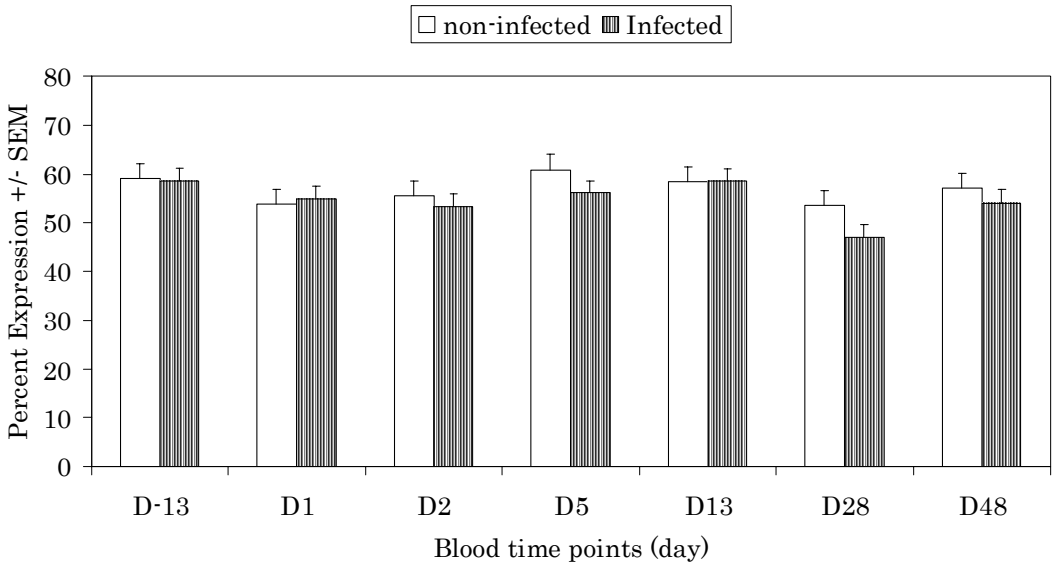

Figure 4.1: Changes in the percentages of CD4 lymphocytes between *S. neurona* challenged and non-challenged horses. The percentages of CD4 lymphocytes were determined via flow cytometry. Blood samples from 8 infected horses and 5 non-infected horses were collected in Florida and shipped to Virginia Tech overnight at 4^0C. Baseline data was day 13 (D-13) prior to infection. D1 meant 1 day post infection. Results were expressed as means ± SEM. No significant differences were seen during these time points.

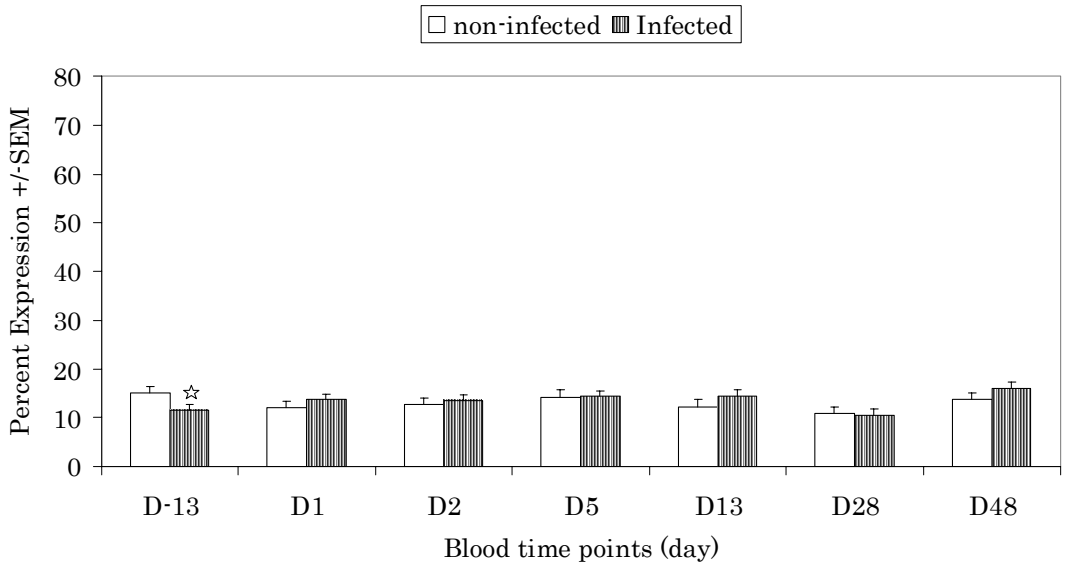

Figure 4.2: Changes in the percentages of CD8 lymphocytes between *S. neurona* challenged and non-challenged horses. The percentage of CD8 lymphocytes were measured via flow cytometry. Blood samples from 8 infected and 5 non-infected horses were collected in Florida and shipped overnight at 4^0C. D-13 meant 13 days prior to infection, and D1 meant 1 day post infection. Values were expressed as means ± SEM. Asterisk indicated a significant difference ($p < 0.05$).

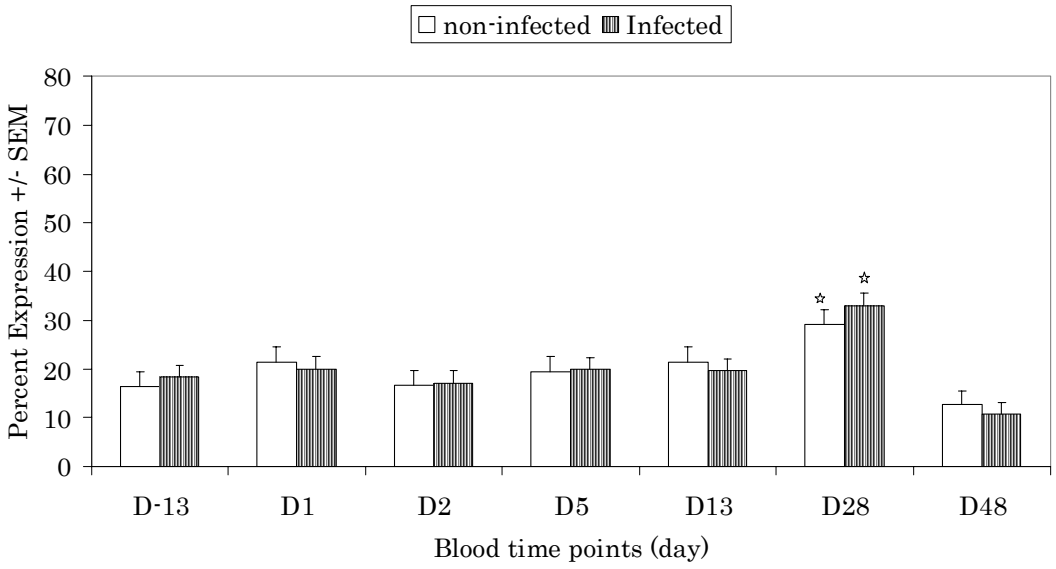

Figure 4.3: Changes in the percentages of B-cell lymphocytes between challenged and non-challenged horses. The percentage of B-cells in PBMCs was measured via flow cytometry. Blood samples from 8 infected horses and 5 non-infected horses were collected in Florida and shipped overnight at 4^0C. D-13 means 13 days prior infection, and D1 means 1 day post infection. Values were expressed as means ± SEM. Values at day 28 post-infection were significantly different from data at other time points. However, no significant differences were present between infected and non-infected horses at day 28 PI. Asterisk indicated a significant difference ($p < 0.05$).

Treatment Effects	Media	ConA (1μg/ml)	ConA (5μg/ml)	PWM (1μg/ml)	PMA/I (20ng/ml vs 10pg/ml)	Live merozoites ($1x10^5$/ml)
Infection Status	0.7954	0.6915	0.3181	0.626	0.2959	0.6685
Date	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0003
Date x Infection Status	0.325	0.8702	0.8146	0.9829	0.0234	0.1334
Neutrophil	0.0051	0.4016	0.3691	0.3526	<0.0001	0.7937

Table 4.1: *P*-values from blastogenesis assays of experimentally infected horses. PBMCs from experimentally infected and uninfected horses were stimulated with media only, mitogens (Final concentrations: ConA at 1 μg/ml and 5 μg/ml, PWM at 1 μg/ml, and PMA/I at 20 ng/ml and 10 pg/ml at the final concentrations) and *S. neurona* merozoites ($1x10^4$/well). All the presented proliferation data were log-transformed to match the selected statistical model. Treatment effects included Date (time points for blood collection) and Infection Status (uninfected and infected groups). Analysis of the interaction between Date and Infection Status reflected the difference between EPM and normal horses at specific time points. Neutrophil was a covariate which reduced the influence of neutrophil contamination on the treatment means. In this way, it increased the power of tests for hypotheses. All the data were log-transformed. Significance was assigned at $p < 0.05$.

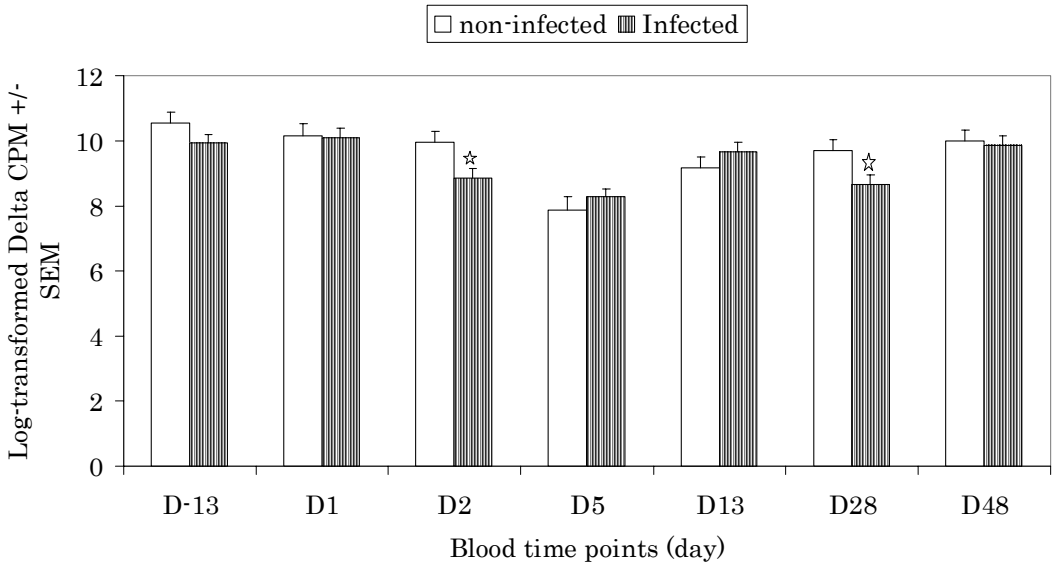

Figure 4.4: Proliferation data from cells stimulated by PMA/Ionomycin in experimentally infected vs control horses. PBMCs from experimentally infected and uninfected horses were stimulated by PMA/Ionomycin. Blood samples from 8 infected horses and 5 non-infected horses were collected in Florida and shipped overnight at 4^0C. D-13 meant 13 days prior infection, and D1 meant 1 day post infection. Original data was log-transformed to fit the statistical model. Values were expressed as means ± SEM. Asterisk indicated a significant difference between infected and non-infected horses ($p < 0.05$).

Mitogen	ConA	ConA	Merozoites	Merozoites
Treatment Effects	CD4	CD8	CD4	CD8
Infection Status	0.4938	0.1531	0.4977	0.7058
Date	<0.0001	<0.0001	<0.0001	<0.0001
Date x Infection Status	0.6362	0.41	0.7657	0.5091
Baseline	<0.0001	0.0353	0.0036	0.793

Table 4.2: *P*-value of cytokine data. Blood samples from 8 infected horses and 5 non-infected horses were collected in Florida and shipped overnight at 4^0C. PBMCs were incubated with either ConA (final concentration 10 μg/ml), *S. neurona* live merozoites ($1x10^4$/well), or media only overnight at 37^0C, 5% CO_2 and separately stained with CD4 or CD8 and IFN-γ antibodies. Cells were measured via flow cytometry to determine the IFN-γ CD4 or IFN- ɣ CD8 double positive cells. Column "CD4" meant the percentage of CD4 cells producing IFN-γ of total lymphocytes. Column "CD8" meant the percentage of CD8 cells producing IFN-γ of total lymphocytes. Treatment effects included Date (time points for blood collection) and Infection Status (uninfected and infected groups). Analysis of the interaction between Date and Infection Status reflected the differences between EPM and normal horses at specific time points. Baseline data was day 13 prior infection. Significance was assigned at $p < 0.05$.

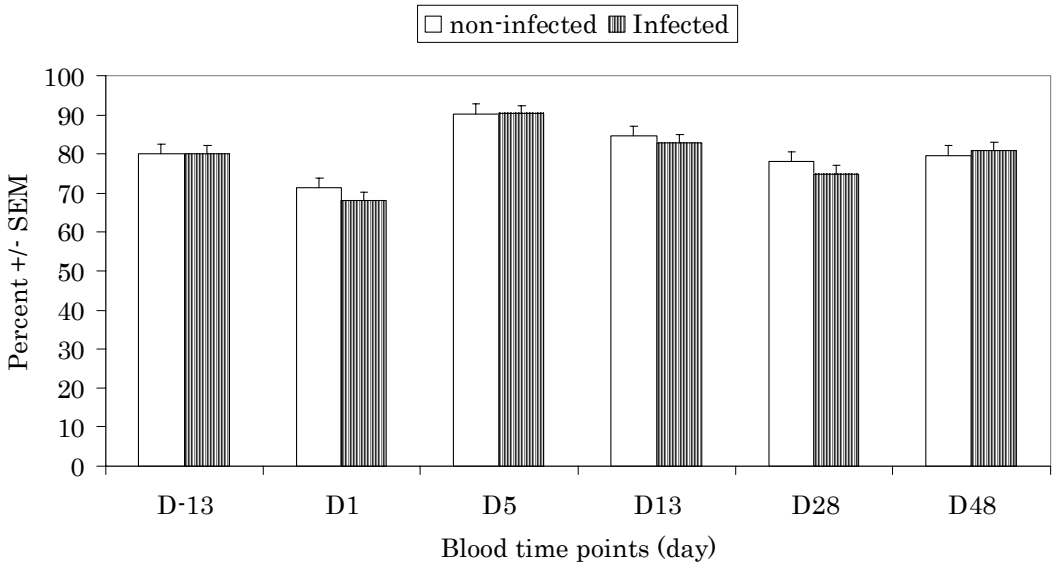

Figure 4.5a: The percentage of IFN-γ positive CD4 cells as a percentage of total CD4 T cells incubated with ConA. Flow cytometry was used to detect the percentage of IFN-γ positive CD4 cells as a percentage of the total CD4 cells. Blood samples from 8 infected horses and 5 non-infected horses were collected in Florida and shipped overnight at 4^0C. PBMCs were incubated with ConA at 5 μg/ml overnight. D-13 meant 13 days prior infection, and D1 meant 1 day post infection. Values were presented as means ± SEM.

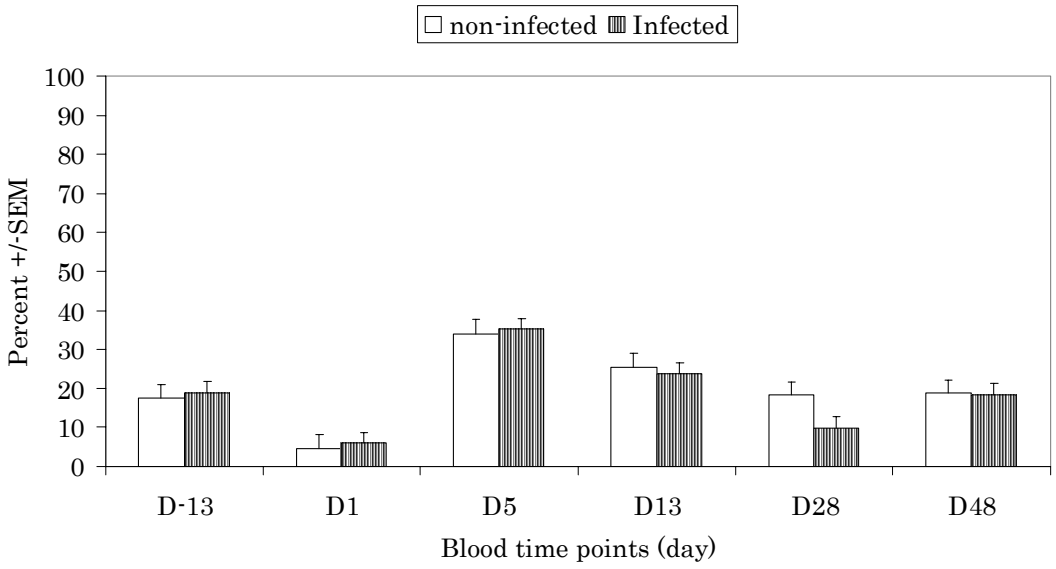

Figure 4.5b: The percentage of IFN-γ positive purified CD4 cells of the total lymphocytes incubated with ConA. Flow cytometry was used to detect the percentage of IFN-γ positive CD4 cells as a percentage of the total CD4 lymphocytes. Blood samples from 8 infected horses and 5 non-infected horses were collected in Florida and shipped overnight at 4^0C. PBMCs were incubated with ConA at 5 μg/ml overnight. D-13 meant 13 days prior infection, and D1 meant 1 day post infection. Values were presented as means ± SEM.

Treatment Effects	CD8 – PMA/I	CD8 + PMA/I	CD8 early apoptosis + PMA/I
Infection Status	0.0175	0.0021	0.0462
Date	0.0013	0.4175	0.041
Date x Infection Status	0.7117	0.336	0.1952
Treatment Effects	PMN early apoptosis – PMA/I	PMN early apoptosis + PMA/I	Monocyte early apoptosis – PMA/I
Infection Status	0.0377	0.1698	0.0744
Date	<0.0001	0.0091	<0.0001
Date x Infection Status	0.0439	0.0459	0.0138

Table 4.3: Selected p-value data for PMA/Ionomycin apoptosis assay. Statistical results with significant differences are presented in the above table for PMA/I apoptosis assay. Blood samples from 8 infected horses and 5 non-infected horses were collected in Florida and shipped overnight at 4^0C at the following time points: day 2, day 5, day 13, and day 48. PBMCs were isolated and incubated with PMA/I (20 ng/ml and 10 pg/ml) or media only overnight. Cells were incubated with primary antibodies CD4, CD8, B-cell, or DH59b followed by FITC/PE second antibodies. 7-AAD was added to detect the degree of cell apoptosis. Five thousand gated events per sample were collected by flow cytometry. Treatment effects included Date (time points for blood collection) and Infection Status (uninfected and infected groups). Treatment groups were CD8 – PMA/I (The percentage of CD8 cells present from lymphocytes incubated without PMA/I); CD8 + PMA/I (The percentage of CD8 cells present from lymphocytes incubated with PMA/I); CD8 early apoptosis + PMA/I (Percentage of CD8 cells undergoing early apoptosis following lymphocyte stimulation with PMA/I); PMN early apoptosis – PMA/I (Percentage of PMNs undergoing early apoptosis following leukocyte stimulation without PMA/I); PMN early apoptosis + PMA/I (Percentage of PMNs undergoing early apoptosis following

leukocyte stimulation with PMA/I); Monocyte early apoptosis – PMA/I (Percentage of monocytes undergoing early apoptosis following leukocyte stimulation without PMA/I). Analysis of the interaction between Date and Infection Status reflected the difference between EPM and normal horses at specific time points. Significance was assigned at $p < 0.05$.

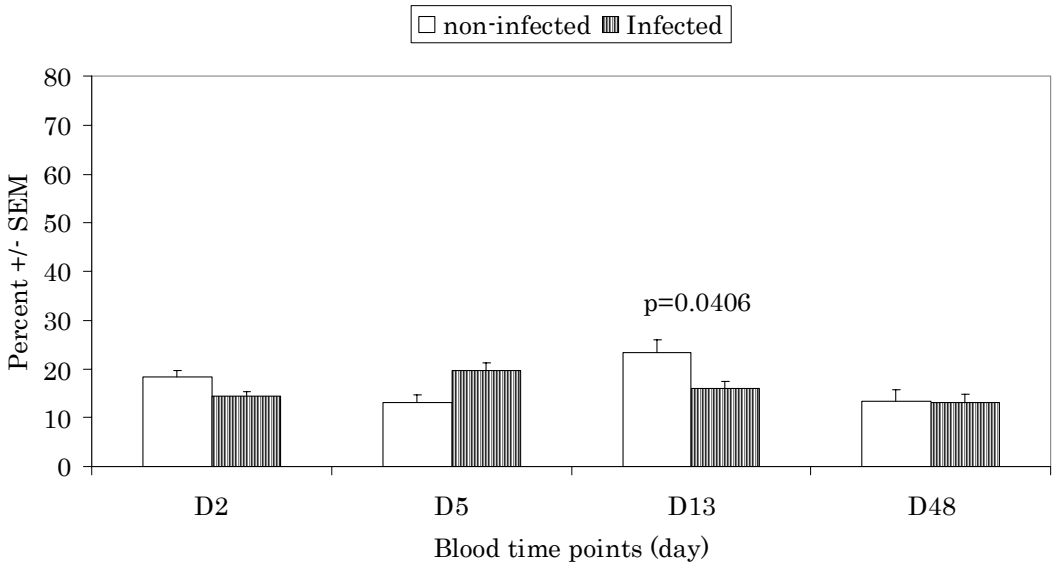

Figure 4.6: Percentage of CD8 cells in early apoptosis of the total CD8 cells when incubated with PMA/I (p = 0.1952). Blood samples from 8 infected horses and 5 non-infected horses were collected in Florida and shipped overnight at 4^0C. Isolated PBMCs were incubated with PMA/I overnight. Cells were stained with CD8 Abs and 7-AAD was added to detect the degree of apoptosis before samples were measured via flow cytometry. D2 meant 2 days post infection. Values were expressed as means ± SEM. The p-value 0.1952 represented the interaction between Date and Infection Status.

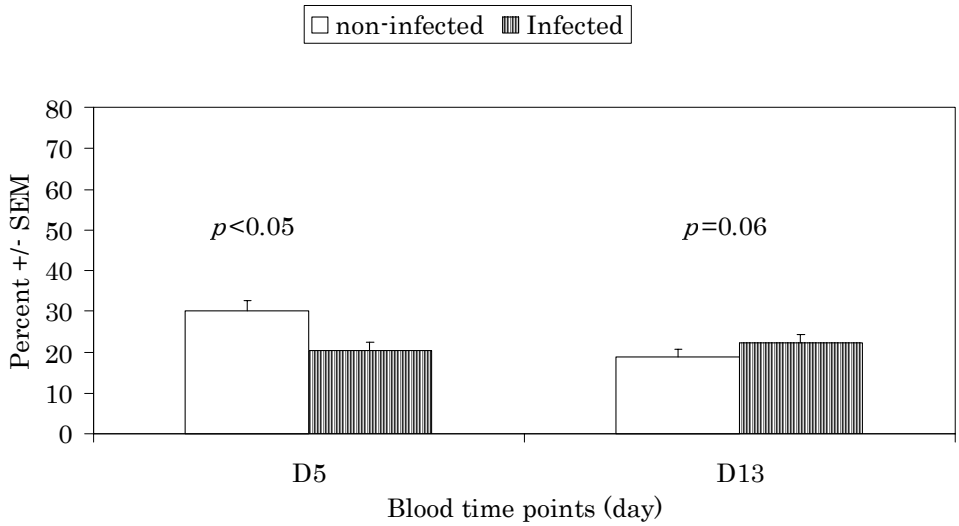

Figure 4.7a: Percentage of CD8 cells following overnight stimulation with ConA ($p < 0.05$). Flow cytometry was used to detect the percentage of CD8 cells of lymphocytes. Blood samples from 8 infected horses and 5 non-infected horses were collected in Florida and shipped overnight at 4^0C. PBMCs were incubated with ConA at 5 μg/ml overnight. D5 meant 5 days post infection, and D13 meant 13 days post infection. The presented data were the representive data based on 6 time points. Values were presented as means ± SEM.

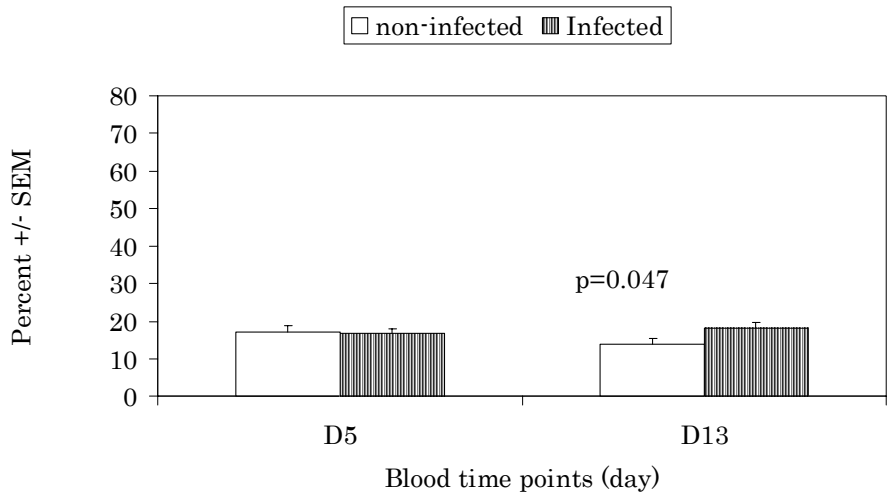

Figure 4.7b: Percentage of CD8 cells following overnight stimulation with merozoites (p = 0.084). Flow cytometry was used to detect the percentage of CD8 cells of lymphocytes. Blood samples from 8 infected horses and 5 non-infected horses were collected in Florida and shipped overnight at 4^0C. PBMCs were incubated with ConA at 5 μg/ml overnight. D5 meant 5 days post infection, and D13 meant 13 days post infection. The presented data were the representive data based on 6 time points. Values were presented as means ± SEM. The p-value 0.084 represented the interaction between Date and Infection Status.

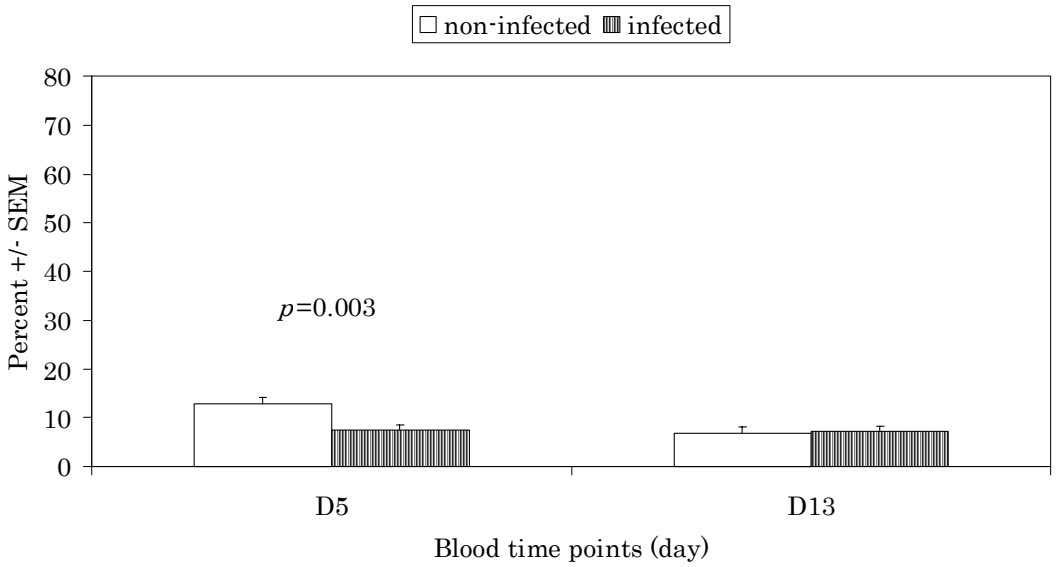

Figure 4.7c: Percentage of CD8 cells producing IFN-γ following overnight stimulation with ConA (*p* = 0.10). Flow cytometry was used to detect the percentage of IFN-γ positive CD8 cells as a percentage of the total lymphocytes. Blood samples from 8 infected horses and 5 non-infected horses were collected in Florida and shipped overnight at 4^0C. PBMCs were incubated with ConA at 5 μg/ml overnight. D5 meant 5 days post infection, and D13 meant 13 days post infection. The presented data were the representive data based on 6 time points. Values were presented as means ± SEM. The *p*-value 0.10 represented the interaction between Date and Infection Status.

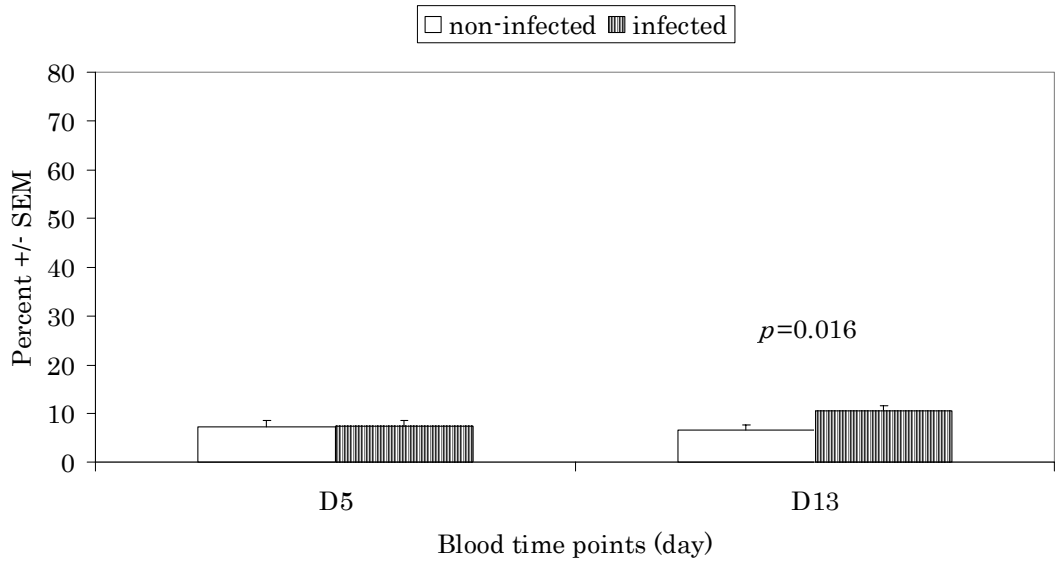

Figure 4.7d: Percentage of CD8 cells producing IFN-γ following overnight stimulation with merozoites (*p* = 0.06). Flow cytometry was used to detect the percentage of IFN-γ positive CD8 cells as a percentage of the total lymphocytes. Blood samples from 8 infected horses and 5 non-infected horses were collected in Florida and shipped overnight at 4^0C. PBMCs were incubated with ConA at 5 μg/ml overnight. D5 meant 5 days post infection, and D13 meant 13 days post infection. The presented data were the representive data based on 6 time points. Values were presented as means ± SEM. The *p*-value 0.06 represented the interaction between Date and Infection Status.

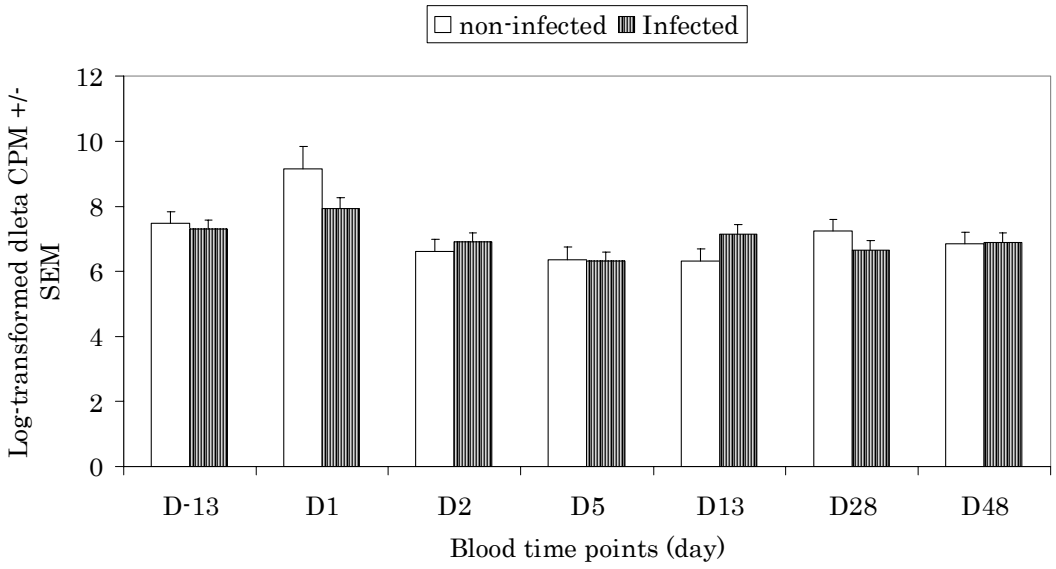

Figure 4.8a: Proliferation data from cells stimulated by *S. neurona* merozoites in experimentally infected vs. control horses. PBMCs from experimentally infected and uninfected horses were stimulated by *S. neurona* merozoites. Blood samples from 8 infected horses and 5 non-infected horses were collected in Florida and shipped overnight at 4^0C. D-13 meant 13 days prior infection, and D1 meant 1 day post infection. Original data was log-transformed to fit the statistical model. Values were expressed as means ± SEM. Asterisk indicated a significant difference between infected and non-infected horses ($p < 0.05$).

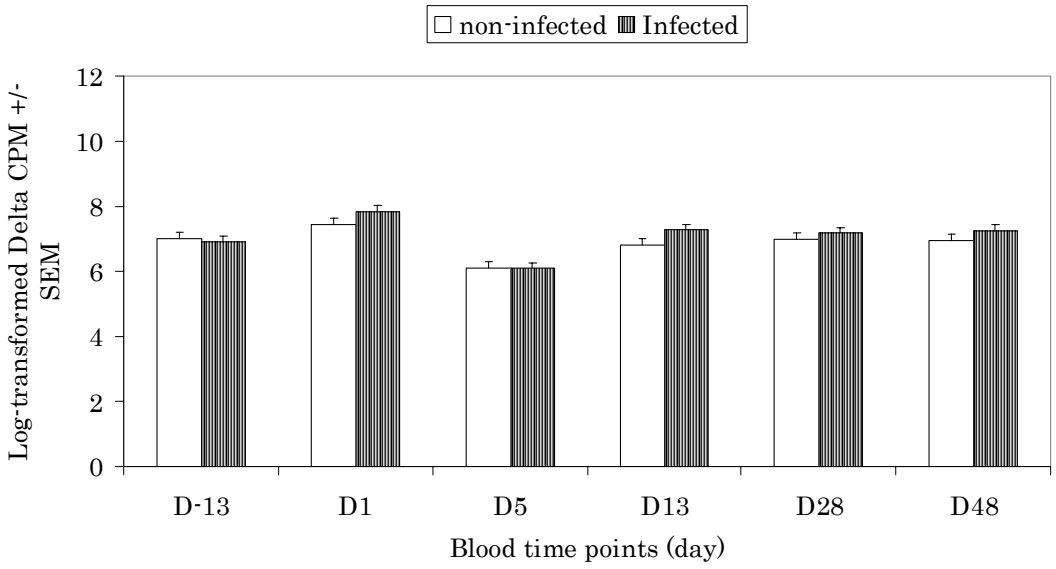

Figure 4.8b: Proliferation data from cells stimulated by SAG-1 in experimentally infected vs. control horses. PBMCs from experimentally infected and uninfected horses were stimulated by SAG-1. Blood samples from 8 infected horses and 5 non-infected horses were collected in Florida and shipped overnight at 4^0C. D-13 meant 13 days prior infection, and D1 meant 1 day post infection. Original data was log-transformed to fit the statistical model. Values were expressed as means ± SEM. Asterisk indicated a significant difference between infected and non-infected horses ($p < 0.05$).

Chapter 5 General Summary and Conclusions

These studies investigated how *S. neurona* altered host immune responses in naturally and experimentally infected horses. First of all, in order to determine whether PBMCs from shipped or stored blood could accurately be utilized to measure cellular immune response in horses, we compared the changes in immune cell subsets and immune function between day 0 and day 1 samples. We found that there were no significant differences in lymphocyte subsets from overnight refrigerated or shipped blood samples compared to day 0, except for the CD8 population, which was significantly decreased. This was in agreement with recently published results by Witonsky et al. (2003c). In addition, overnight storage or shipping of blood samples did affect PWM stimulated responses and *S. neurona* merozoite stimulated responses. Furthermore, we observed that temperature could significantly affect the immune cell subsets and proliferation results of both normal and *S. neurona* infected samples. This point was demonstrated by the increased percentage of neutrophils in PBMCs from both EPM and normal horses if blood samples were less than 4^0C. PMA/I stimulated proliferation response decreased considerably when neutrophils contaminated the PBMC population. If efforts were not made to assess temperature and recovery, results may not accurately reflect immune function.

Once potential differences in immune function based on with storage conditions were determined, efforts focused on determining the effects of infection. There appeared to be an increased percentage of CD4 cells of lymphocytes in naturally

infected EPM horses (day 0 only) that we did not observe in experimentally infected horses (day 1 only). By comparison, Tornquist, et al. (2001) reported a decreased percentage of CD4 cells in naturally infected horses of EPM. The results from these studies further suggested that the selected horse population (either horse breed and age or *S. neurona* infection factors) in each study could significantly influence the results. This was more likely due to significant variation with respect to time since infection, dose of organism, severity of disease and other related conditions within the existing selected population of EPM horses employed for these different studies. For definitive conclusions to be reached with regard to CD4 levels, it would be ideal to perform a more extensive study over a prolonged period of time that would include more animals with similar ages, location, duration, and severity of disease, and include both day 0 and day 1 samples.

Both the studies with naturally and experimentally infected horses detected some changes in immune function associated with *S. neurona* infection. Naturally infected horses showed a decrease, which was not statistically significant, in the specific immune response to *S. neurona* merozoites in day 0 samples. This was in agreement with the studies by Tornquist et al. (2001) and Spencer et al. (2004). In addition to changes in merozoite stimulated responses, PMA/I–stimulated PBMCs from both naturally and experimentally infected horses were suppressed compared to normal horses. The mechanism for this will be addressed in future studies.

Besides assessing immune function based on proliferation, IFN-γ cytokine production was also measured in experimentally infected horses. Our current

cytokine data did not indicate that *S. neurona* infected horses had a decreased Th1 or Tc1 immune response, even though there was a trend in suppressed CD8 Tc1 immune response at day 5 PI. This may be due to the early time points of sample collection, severity of disease, or experimental model. Alternatively, it could be that there were limitations with the method and conditions we employed to detect the presence of IFN-γ positive CD4/CD8 cells in response to ConA or *S. neurona* merozoites. We did not measure total IFN-γ production. We propose to develop and use an ELISA in our future studies to quantify the amount of IFN-γ produced by each CD4/CD8 cell when cells are stimulated with ConA or *S. neurona* merozoites, and to assess other contributing populations like monocytes.

In conclusion, our results did not support that EPM horses had lower CD4 lymphocytes compared to neurologic normal but seropositive or seronegative horses. However, EPM horses showed decreased proliferation responses in the PBMCs following PMA/I stimulation, which suggests that *S. neurona* infection could alter the signaling pathway of PMA to activate cell proliferation. We also compared the effect of day on immune cell subsets and immune function, which offered reference for people to include day 1 samples in the EPM studies. In our hypothesis, we proposed that EPM horses had suppressed cell-mediated immune response compared to neurologically normal seropositive horses. Our current data did not support the hypothesis directly. However, data from intracellular IFN-γ staining suggested that EPM horses could have suppressed CD8 Tc1 immune responses in the early infection stage (day 5 PI). The suppressed CD8 Tc1 decreased IFN-γ production which was critical to prevent *S. neurona* mediated encephalitis in mice.

Future studies on the role of IFN-γ are needed to learn of immunity to *S. neurona* in horses. This may provide a better understanding of the mechanisms of how horses develop neurologic disease. To develop neurologic signs in any horse, *S. neurona* sporocysts must penetrate the vascular endothelium of the BBB into the immune privileged CNS, where they survive. An *in vitro* study by Lindsay (unpublished data) indicated that *S. neurona* merozoites preferred to infect monocytes when incubated with PBMCs. As we know, monocytes are an important IFN-γ- producing cell, and IFN-γ is critical to protection against *S. neurona*. We question whether IFN-γ has a role in the prevention of *S. neurona* infection of the monocytes. If *S. neurona* somehow suppresses IFN-γ production in monocytes or other cell types (CD4, CD8), it would appear that this would be a means by with *S. neurona* could gain access to the CNS. Future studies could focus on determining whether IFN-γ production is suppressed in multiple cell types, and whether IFN-γ production is critical to prevention of *S. neurona* infection of monocytes.

Overall, this study provides a foundation for *in vitro* studies on equine immune function associated with *S. neurona* infection. A considerable amount of knowledge is still needed before the immunopathogenesis of EPM is elucidated.

FUTURE STUDIES

Future studies should involve investigation into possible mechanisms associated with the decreased proliferation response to PMA/I in EPM affected horses. Possible explanations, which appear most likely at this time, include changes in lymphocyte subpopulations present between EPM and normal horses and altered signaling in the PMA/I pathway associated with the *S. neurona* infection. An alteration in the signaling pathway for PMA/I could be the most important reason for the decreased proliferation response to PMA/I in EPM horses. To determine whether *S. neurona* activates the PMA pathway upon infection of a cell, future interests should center on intracellular signaling mechanisms involved in lymphocyte activation by PMA/I. Since PKC plays an important role in T-cell activation (Richards et al., 2004), it suggests that the activity or concentration of intracellular PKC isoenzymes might be inhibited by *S. neurona* infection, which alters the signaling of the PMA/I pathway. Future studies can measure the activity or concentration of tyrosine kinases (i.e. MEK, ERK), other signaling molecules (i.e Raf), intracellular protein kinase C isoenzymes, and MAPK in the PMA signaling pathway of *S. neurona* infected equine lymphocytes.

In addition, the lymphocyte subpopulations stimulated by PMA/I are potentially different from those stimulated by ConA and PWM. It is critical to figure out which equine lymphocyte subpopulations PMA/I stimulates. We may incubate each magnetically or sorted enriched subpopulations such as CD4 cells, CD8 cells, B-cell, and monocytes separately with PMA/I to see the differences of the proliferation

rates among each subpopulation. Alternatively, we could look at differences in proliferation profiles by staining cells with CFSE prior to adding PMA. It would be helpful to look at functional differences in certain populations like CD4 T cells (IFN-γ vs IL-4 production) as well as activation status of particular subtypes. Differences in apoptosis could be assessed by 7-AAD staining and analysis by flow cytometry. Additional experiments could be designed to utilize 5-bromo-2-deoxyuridine (BrdU) and cell surface marker antibodies to detect which subpopulations proliferate in response to stimulation with PMA/I.

In order to accurately assess IFN-γ production, suggestions for further optimization of this approach include: measuring the amount of IFN-γ production via ELISA, prolonging incubation times with merozoites/SAG-1, or collecting blood samples for longer time points following infection. Since there were limited SAG-1 data for statistical analysis in both naturally and experimentally infected horses, optimization of SAG-1 assay would be necessary. It may be better to increase the concentration of SAG-1, or incubation time. Regarding antigen specific proliferation stimulated with *S. neurona* merozoites, optimization of the merozoite concentration, incubation time, or cell concentration would be necessary in future studies. Once these conditions have been optimized and samples were analyzed utilizing these methods, more rewarding information may be gained.

In summary, with the use of the reproducible equine model of EPM, this study is just the first of many which is needed to address the many questions regarding the protective and pathophysiologic immune responses to *S. neurona* infection in horses.

In this study, we have consistently demonstrated a suppressed *in vitro* proliferation response to PMA/I in both naturally and experimentally infected EPM horses. Once the mechanisms of suppression are elucidated, the significance of these findings in the overall pathophysiology of disease will be better understood. Additional studies will focus on addressing potential mechanisms of immunsuppression. Hopefully, through united efforts, we will ultimately be more success in diagnosing, treating and preventing this devastating disease.

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