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THE EFFECTS OF OVINE INTERFERON-τ TREATMENT ON THEILER'S VIRUS-INDUCED DEMYELINATION IN FEMALE SJL/J MICE

A Dissertation

by

KASANDRA TANEESHIA TRAWEEK

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2001

Major Subject: Veterinary Anatomy

The views expressed in this article are those of the author and do not reflect the official policy or position of the United States Air Force, Department of Defense, or U.S. Government.

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C. Jane Welsh
(Chair of Committee)

Ellen W. Collisson
(Member)

Ralph Storts
(Member)

Rajesh C. Miranda
(Member)

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ABSTRACT

The Effects of Ovine Interferon-τ Treatment on Theiler's Virus- Induced Demyelination in SJL/J Mice. (August 2001)

Kasandra Taneeshia Traweek, B.S., Humboldt State University; M.A., University of the Incarnate Word

Chair of Advisory Committee: Dr. C. Jane Welsh

Theiler's murine encephalomyelitis virus (TMEV) is a picornavirus that is used as an animal model for the study of multiple sclerosis (MS). One of the current therapies for MS involves the administration of a type I interferon, IFN-β. The administration of IFN-β can result in unfavored, psychological side-effects including clinical depression. A second type I interferon, ovine IFN-τ, has previously been found to be less cytotoxic than IFN-β. This study focuses on the effects of IFN-τ on SJL/J mice at 0 months, 2 months, and 4 months post-infection with TMEV strain BeAn. Through neurological exams and histological analysis of the CNS, we report that IFN-τ administration significantly reversed the neurological abnormalities induced by TMEV infection when given at 4 months post-infection. Histologically, treatment at 4 months post-infection resulted in a significant decrease in lesion occurrence in the ventral and dorsal funiculi of the spinal cord. Inflammation and demyelination were both reduced as measured via percent area effected in hemotoxylin + eosin stained and myelin oligodendrocyte glycoprotein-specific immunostained spinal cord sections embedded in paraffin. No effect was detected with treatment at 2 months post-infection. In contrast, SJL/J mice pre-treated with IFN-τ displayed increased disease progression when compared to controls, as well as increased lesion

development. Treatment with IFN-τ from 4 to 6 months post-infection resulted in an increase in serum IL-10 levels, increase in IL-1, IL-6, IL-10, and IFN-γ mRNA in the spinal cord, and a decrease in delayed-type hypersensitivity response to myelin membrane when compared to saline treated, infected controls. No significant change was noted in white blood cell differential between IFN-τ or saline treated infected animals. In conclusion, it is proposed that IFN-τ mediates its therapeutic effects by upregulating IL-10 which suppresses delayed-type hypersensitivity responses thereby inhibiting the inflammatory demyelinating process. IFN-τ may be a useful therapeutic agent in the treatment of multiple sclerosis.

DEDICATION

The work outlined in this dissertation is by no means the efforts of one person. There have been many special people in my life who have believed in and supported me whenever I found it difficult to believe in myself. This work was not possible without the strong foundation the following people have set for me over the years. Thank you for your love and encouragement.

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to continue my work because I respect her greatly as a researcher and friend. Thank you, Dana.

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I. INTRODUCTION

Background

1.1 Multiple sclerosis

One of the major demyelinating diseases of the central nervous system (CNS) is multiple sclerosis (MS). In addition to demyelination of the white matter, MS symptoms also result from inflammation characterized by the infiltration of lymphocytes and macrophages into the CNS, truncated or transected axons, and deterioration of neurological function (Prineas and Raine, 1976; Prineas and Wright, 1978; Adams et al., 1989, Trapp et al., 1998). To clinically diagnose MS for laboratory research purposes, samples of cerebrospinal fluid are analyzed for: free and bound antibodies to myelin basic protein (MBP), proteolipid protein (PLP), or myelin oligodendrocyte glycoprotein (MOG), as well as other myelin-autoreactive B- and T-cell responses, such as the upregulation of Th1 cytokines (Warren and Catz, 1993; Olsson, 1994; Warren and Catz, 1994).

There are currently over 300,000 cases of MS documented in the US. Women are affected twice as often as men with the typical onset between the ages of 20-40 years. Patients are characterized with relapsing-remitting (RR) or primary-progressive (PP) MS at diagnosis (Lublin et al., 1996). Those affected by RR MS experience serial periods of neurological dysfunction that increase in severity over time followed by brief periods of recovery. PP patients experience a steady gradual decrease in neurological function. Approximately 40% of RR patients eventually develop another form of MS, secondary-progressive, that mimics the PP form.

This dissertation follows the style of the Journal of Neuroimmunology.

MS lesions can differ considerably between patients depending on the disease form that is diagnosed. The etiology of MS is unknown, but individual variation in lesions that develop as well as multiple immunogenetic factors have raised the possibility that MS may be the result of several different disease processes (Ebers et al., 1996; Haines, et al., 1996; Sawcer et al., 1996). A recent report by Lucchinetti et al. suggests two different patterns of lesion development, one that is T-cell-mediated or T-cell plus antibody-mediated autoimmune encephalomyelitis and the other involving primary oligodendrocyte damage as the result of a viral or bacterial infection. Of the 83 patients examined, all were found to have both types of MS lesions though multiple active lesions within the same patient were found to involve only one type (Lucchinetti et al., 2000). The presence of two different lesion patterns support a multifactorial etiology.

1.2 Major histocompatability complex genes

In humans, the major histocompatability complex (MHC) genes are located on the short arm of chromosome 6 whereas murine MHC genes are located on chromosome 17 of mice. The MHC genes are divided into three different classes, MHC class I, II, and III. MHC class I genes are further subdivided into MHC class Ia and Ib. MHC class Ia genes are highly polymorphic where as class Ib genes are not. In humans, there are three class Ia loci: A, B and C. The class Ia loci in the mouse H-2 complex are K and D. In human populations, as many as 23 α chain alleles can be found at the A locus, 49 α chain alleles at B, and 8 α chain alleles at C. This polymorphism is a result of variations in the amino acid sequence of the α1 and α2 domains of the MHC class I

molecule. The MHC class II genes are also polymorphic. To date, three MHC class II loci, DR, DQ, and DP have been reported with a possibility of 19 DR alleles, 7 DQ alleles, and 7 DP alleles. The MHC class III genes are not directly liked to antigen presentation, but do play a role in immunity through the production of complement serum proteins (Trowsdale, 1995).

Cytotoxic T-cells (CTLs), T-helper 1 (Th1), and T-helper 2 (Th2), cells all play a role in the development of inflammation in the cell-mediated and antibody-mediated immunological responses. CTLs are activated by the expression of $CD4^+$ cytokines IL-2, IL-4, and IL-6 (Maliszewski et al., 1990). CTLs are able to lyse cells that express foreign antigen in MHC class I molecules causing the production of cellular debris that is phagocytosed by macrophages. Extensive macrophage infiltration and burst can result in inflammation.

Th1 cells will secrete IL-12, IL-2, interferon-gamma (IFN-γ), and tumor necrosis factor-beta (TNF-β) when a $CD4^+$ T-cell detects a foreign antigen within MHC class II on an antigen presenting cell. IFN-γ release activates macrophages which result in increased MHC class II expression, increased phagocytosis and infiltration of macrophages to the site of injury. This is primarily seen with delayed-type hypersensitivity reactions (DTH) (Bottomly, 1988; Altman et al., 1990). IL-12 stimulates the differentiation of primary T-cells (Th0) to Th1 cells as well as $CD8^+$ T-cells to mature and active CTLs. IL-2 stimulates natural killer cells (NK), that also produce IFN-γ and prime macrophages. TNF-β promotes B-cell proliferation and immunoglobulin secretion, specifically IgM and IgG2a antibodies.

The interleukins produced by Th2 cells: IL-4, IL-5, IL-6, IL-10 and IL-13 upregulate B-cell proliferation and the production of specific polyclonal immunoglobulins IgG1, IgA, IgM, and IgE (Williams, 1984).

The Th2 interleukins all down regulate the IFN-γ/NK cell/macrophage activation pathway and enhance MHC class II expression (Seder and Le Gros, 1995). Th2 cells can inhibit T-cell mediated inflammation and suppress cytokine production in macrophages. In chronic inflammation, the proliferation of Th2 cells is beneficial as they reduce the volume of inflammatory cells that accumulate at the site of injury (Weissmann et al., 1980).

1.3 Multiple sclerosis immunology

Cytokine expression plays a role in the development of demyelinating lesions in MS patients. In order for the MS lesions to occur, myelin self-reacting $CD4^+$ Th1 cells can be activated to release proinflammatory Th1 cytokines IL-2, IFN-γ and TNF-β (Bottomly, 1988), must travel from the circulatory system into the CNS (Martin and McFarland, 1995; Lucchinetti and Rodriguez, 1997). T-cells collected from MS patients during relapsing episodes are known to produce the Th1 cytokines: IFN-γ, TNF-α, and TNF-β, (Correale et al., 1995; Voskuhl et al., 1993; Olsson et al., 1990). Conversely, the Th2 response involves the secretion of anti-inflammatory cytokines such as IL-4 and IL-10 that are prevalent in MS patients during remission phases (Correale et al., 1995). Through the secretion of IFN-γ, Th1 cells aid in the switch in immunoglobulin production from IgM to IgG2a as well as inhibit Th2 lymphocyte cytokine production. In comparison, Th2 cells facilitate the IgG1 switch to IgE and indirectly suppress Th1 lymphocytes by affecting antigen presenting cells via IL-4, IL-5, and IL-6 (Finkelman, et al., 1990; Mosmann and Coffman, 1989). The possibility that Th1 cytokines may increase the severity of MS lesions is further supported by observation that IFN-γ

administration to MS patients results in the worsening of symptoms (Panitch et al., 1987).

1.4 Multiple sclerosis: genetic and environmental factors

The etiology of MS is still unknown though epidemiological studies of the worldwide distribution of MS suggest an infectious agent is involved. The highest prevalence of MS, with 30 per 100,000 cases diagnosed per population, occur in northern Europe into the Union of Soviet Socialist Republics, southern Canada, and northern United States, New Zealand, and southeastern Australia. Scandinavia, central Union of Soviet Socialist Republics, southern Europe, southern United States, and much of Australia are considered intermediate risk areas. Finally, Asia and the tropics appear to be the lowest frequency areas with occurrence rates below 5 per 100,000 in the population. (Goldberg and Kurland, 1962; Kurtzke, 1980; Massey and Schoenberg; 1982). It is important to note that the above occurrence rates appear to be correlated with the location where the patient lived before the age of 20. If the patient moved before that age, the environmental risk level shifts to the new location (Kurtzke, 1993).

It has been proposed that genetics play some role in the development of MS. Though it is improbable that there is one gene responsible for susceptibility to MS, it has been documented that some human leukocyte-associated (HLA) genotypes render people more susceptible to autoimmune disease. In MS, no HLA subset has thus far been found to be elevated in all MS patients assayed, but HLA-A3 and HLA-B7, two of the MHC class I genes, have been associated with the prevalence of MS in northern European whites. MHC class II genes HLA-DR2 and/or HLA-DW2 or HLA-DR4 have also been suspected for

the Northern European whites. An elevated HLA-DR2 expression in MS patients has been the most promising gene related to this disease since it has been seen in the United States, Europe, South Africa, and Mexico (Gorodezky et al., 1962; Fewster and Kies, 1984; Sriram et al., 1985; Van Lambalgen et al., 1986). Patients in Scotland were found to have excess HLA-DQw1 and HLA-DQβ, instead of HLA-DR2 (Francis et al., 1983; McDonald, 1984; Marcadet et al., 1985). Kolstad et al. discovered that HLA-DQβ was expressed in 34 of the 35 MS patients they genotyped, though such a high correlation has yet to be repeated (Kolsted et al., 1989). In short, it appears that MHC genetics may play a role in MS, but it is certainly not the only factor to consider.

1.5 Possible viral etiology for MS

Based on the geographic rates of MS development and risk of disease due to migration, it has been postulated that a viral infection before CNS maturity may play a role in MS (Martin and McFarland, 1995). It is possible that a viral antigen may mimic a host protein thereby initiating an autoimmune reaction that results in chronic inflammatory disease (Rose, 1994; Tsunoda and Fujinami, 1996). One specific viral infection has not been found consistently in all MS patients examined post-mortum, but several viral pathogens have been proposed due to high titers that have been detected in some MS patients when compared to controls. Measles, human coronavirus 226E, Epstein-Barr virus, retroviruses, and human herpes virus-6 have all been extracted from the CNS or cerebrospinal fluid of MS patients (Cook et al., 1996).

1.6 MS and pregnancy

A modification of the maternal immune system is necessary during pregnancy in order to prevent spontaneous abortion. The intermediate metabolites produced when cholesterol is transformed into one of the sex hormones, progesterone, testosterone, and estrogens, are able to interact with the immune system (Lahita et al., 1981; Sambrook et al., 1988). The production of immunosuppressive proteins during pregnancy include increased progesterone (Siiteri et al., 1977), prolactin (Eriksson et al., 1989), estrogen (Lahita, 1992) and interferon-tau (IFN-τ), in sheep (Soos et al., 1995). The placenta is able to produce IL-10 (Wegmann et al., 1993; Raghupathy, 1997) and suppress maternal MHC-specific T cells that recognize the fetus as non-self (Tafuri et al., 1995).

Women with RR MS experience approximately a 70 percent decrease in the rate of relapse during the third trimester of pregnancy as compared to their disease progression a year before pregnancy. This protective effect dissipates within the first three months post-partum. Relapsing rates appear to increase during the first year post-partum (Poser and Poser, 1983; Confavreux et al., 1998), but over-all long term increase in disease progression due to pregnancy has not been found (Thompson et al., 1986; Weinshenker et al., 1989; Roullet et al., 1993). There is an apparent shift from a predominant Th1 reaction to a Th2 response during pregnancy, which is reversed after birth of the fetus (Wegmann et al., 1993). Since the Th1 response plays a pathogenic role during MS, the Th2 activation in the mother during the last trimester may account for the remitting period experienced during pregnancy.

1.7 Interferon-beta treatment of MS

Interferon-beta (IFN-β) has a therapeutic effect in MS patients by reducing the severity of MS symptoms and relapse rate (Paty and Li, 1993; Jacobs et al., 1996). However, several undesirable side effects have been noted. Fever, myalgia, headache, fatigue, and chills occur in 75% of treated patients though the duration of these side-effects is patient specific (Lublin et al., 1996). This may be due to the immediate upregulation of inflammatory cytokines and prostaglandins (Arnason and Reder, 1994; Dayal et al., 1995; Brod et al., 1996) resulting in increased spasticity and temporary worsening of symptoms (Lublin et al., 1996).

IFN-β has recently been shown to be effective in the treatment of relapsing-remitting MS (IFN-β Multiple Sclerosis Study Group, 1993). IFN-β also inhibits the progression of relapsing-remitting experimental allergic encephalomyelitis in mice, another animal model for MS (Yu et al., 1996). The exact mechanism of IFN action in these conditions is not completely understood. IFN-β has many diverse biological roles ranging from anti-viral to immunomodulatory effects (Belardelli and Gresser, 1996). Since MS is thought to be initiated by a viral infection, the effectiveness of IFN-β may be related to its role in viral inhibition. However, the autoimmune aspects of MS may be down regulated through a number of different mechanisms since type I IFNs have been shown to inhibit DTH responses (also important in Theiler's virus-induced demyelination (TVID) and EAE), alter the homing and trapping of lymphocytes, enhance NK and T-cell activity and cytotoxicity, enhance Fcγ receptors effects (Belardelli and Gresser, 1996), inhibit T-cell proliferation, and decrease the production of IFN-γ (Noronha et al., 1993). Recently,

IFN-β has been shown to decrease the migration of T-lymphocytes in vitro by interfering with the production of T-cell matrix metalloproteinases which mediate T-cell migration through matrices. Both NK cells, $CD8^+$ and $CD4^+$ T-cells were also affected by this treatment. The authors demonstrated that IFN-β decreased the matrix metalloproteinase levels which in turn prevented the cleavage of fibronectin (Stuve et al., 1996).

1.8 Corticosterone treatment of MS

Corticosterone and cortisol are glucocorticoids that are secreted by the adrenal cortex. Both hormones are products of cholesterol catabolism and they differ by one hydroxyl group. Healthy humans normally produce a 1:5 ratio of corticosterone to cortisol whereas mice only produce corticosterone.

Multiple sclerosis patients are known to have an exaggerated cortisol release when relapsing-remitting MS progresses into secondary progressive MS. This is attributed to proinflammatory cytokines impairing normal glucocorticoid function and the increase in neuronal damage (Bergh et al., 1999). This theory is supported by the fact that neuroendrocine over-activation is seen in MS patients with acute CNS inflammation (Fassbender et al., 1998).

Glucocorticoid therapy can be beneficial in MS treatment, but effectiveness is dependent on the stage of disease progression. Treatment of MS patients with low-dose corticosteroids, 10-20 mg of prednisolone daily, had no significant effect on MS progression with chronic progressive or relapsing-remitting MS (Tourtellotte and Haerer, 1965; Millar et al., 1967). In contrast, rapid clinical improvement is seen in MS patients in acute relapse who have been treated intravenously with methylprednisolone ranging from 250 mg daily to 20

mg/kg daily for 2-7 days (Dowling et al., 1980; Buckley et al., 1982; Abbruzzese et al., 1983; Goas et al., 1983; Newman et al., 1983; Barnes et al., 1985; Durelli et al., 1986; Milligan et al., 1987; Thompson et al., 1989).

The clinical benefits seen in patients with glucocorticoid therapy is attributed to the immunosuppressive effects of corticosterone that are dosage dependent. Supraphysiological corticosteroid dosage can inhibit antibody-dependent cellular cytotoxicity (Nair and Schwartz, 1984), neutrophil complement and IgG receptor binding (Forslid and Hed, 1982), antigen presentation by monocytes (Dumble et al., 1981; Hirschberg et al., 1982; Rhodes et al., 1986), astrocyte binding to measles-infected cells (Maki et al., 1980), monocyte cellular differentiation (Rinehart et al., 1982) and leukocyte production of IL-2 and IFN-γ (Arya et al., 1984; Cesario et al., 1986).

Treatment with high corticosterone levels in Lewis rats with chronic EAE also force a down-regulation of IL-2 and IFN-γ (Dowdell et al., 1999) suggesting a common decrease in Th1 cytokine expression as a result of the abnormal corticosterone levels.

1.9 Animal models for multiple sclerosis

Several animal models of multiple sclerosis have been developed to study treatment strategies and pathology of MS in a laboratory setting. There are seven such models that are commonly used in this capacity, most of which involve virally-induced demyelination. These seven models include animal interactions with: Theiler's virus, mouse hepatitis virus, Semliki Forest virus, visna virus, canine distemper virus, plus experimental allergic encephalomyelitis, and chemical induction of demyelination. Infections with Theiler's virus, mouse hepatitis virus,

Semliki Forest virus, experimental allergic encephalomyelitis, and chemically-induced demyelination are murine models whereas infections with visna virus and canine distemper virus are sheep and canine models respectively. The main focus of this work involves the in-depth understanding of Theiler's virus infection and experimental allergic encephalomyelitis in the mouse. Those models will be outlined in detail below. The five other animal models will be discussed here briefly.

Mouse hepatitis virus (MHV) is a coronavirus. It is an enveloped virus that contains a positive sense, single-stranded, non-segmented, polyadenylated, RNA genome (Lai and Stohlman, 1978). Not all strains of MHV cause hepatitis in mice. MHV-4, A59, and JHM are three neurotropic strains of MHV that produce a pronounced CNS disease. MHV is not naturally neuroinvasive so the virus is injected into the cerebellum or the olfactory nerve of mice or rats to induce CNS disease. MHV-4 is extremely neurovirulent and induces an encephalitis that is fatal within the first week of infection. Strains A59 and JHM are avirulent and cause the development of inflammatory demyelination in the brain and spinal cord (Taguchi et al., 1979; Barthold and Smith, 1983; Koolen et al., 1983; Barthold, 1986). Replication of the avirulent strains is confined to developing oligodendrocytes which induce demyelination via oligodendrocyte cell lysis (Beushausen and Dales, 1985; Pasick and Dales, 1991).

Semliki Forest virus (SFV) is an alphavirus in the family *Togaviridae* and is also a positive-sense, enveloped, RNA virus. L10, M9 and A7 are the three most common strains of SFV that are studied. L10 is virulent (Atkins and Sheahan, 1982; Atkins et al., 1990; Smyth et al., 1990; Glasgow et al., 1991; Balluz et al., 1993) while M9 and A7 are avirulent in adult mice (Atkins et al., 1985, 1995; Fazakerley et al., 1993). SFV is neuroinvasive and neurotropic and may induce

demyelination when administered intraperitoneally. A systematic viremia develops and SFV is able to cross the brain-blood barrier by infecting cerebral vascular endothelial cells. L10 then infects neurons and oligodendrocytes where a fatal encephalitis develops after two weeks post-infection (Barrett et al., 1980; Atkins and Sheahan, 1982; Atkins et al., 1990; Smyth et al., 1990; Glasgow et al., 1991; Balluz et al., 1993). The A7 and M9 strains remain at the site of CNS entry and induce a long-term CNS demyelination by infecting oligodendrocytes and not neurons (Atkins et al., 1985, 1994; Fazakerley et al., 1993). The demyelination caused by SFV-A7 and SFV-M9 infection is dependent upon a bystander effect mediated by $CD8^+$ T-cells (Subak-Sharpe et al., 1993).

Visna virus (VV) is a lentivirus of the family *Retroviridae*. It is a lentivirus with an RNA genome that is transformed to DNA which integrates into the host genome (Pyper et al., 1984). In the sheep, visna virus produces a chronic infection when there is a strong host immune reaction present (Georgsson et al., 1987). Replication is restricted to monocytes that are differentiating into macrophages though the virus is able to stay dormant in monocytes until differentiation occurs (Vigne et al., 1994). Inflammatory infiltrates in the CNS of Icelandic sheep infected with visna virus produces lesions in the white matter and cause necrosis of axons and myelin sheaths (Petursson et al., 1976). This type of "early" white matter damage may be seen even years after initial infection. The "late" type of lesion can take up to 11 years post-infection to detect, but results in demyelination in the spinal cord with axon preservation and continuous myelin breakdown similar to the lesions seen in chronic-progressive MS (Georgsson et al., 1982; Georgsson et al., 1987).

Canine distemper virus is a morbillivirus in the *Paramixoviridae* family that is negative, single-stranded, enveloped, and contains RNA (Norrby and Oxman, 1990). CNS demyelination in this model can be induced through intraperitoneal or intranasal infection of dogs and may be non-inflammatory or inflammatory depending upon the degree of immunosuppression at the time of infection. Non-inflammatory, demyelinating plaques are common in the initial stages of viral infection and are attributed to direct viral effect (Cerruti-Sola et al., 1983). Viral infection of astrocytes is common whereas oligodendrocyte infection is rare and restricted meaning that viral RNA is detected in oligodendrocytes but viral proteins are not. The death of the astrocyte leads to non-viable conditions for the oligodendrocytes due to the increase of extracellular waste products (Summers et al., 1987).

There are several chemicals that may be used to induce CNS demyelination in experimental animals. Some of these chemicals include cuprizone, ethidium bromide, hexachlorophene, lysolecithin, tellurium, and triethyltin. Cuprizone and ethidium bromide can cause status spongiosus of the white matter and intramyelinic edema when administered into the CNS. Hexachlorophene and triethyltin induce brain swelling as well as edema between the myelin sheaths. Lysolecithin is more selective since demyelination occurs only at the site of injection. Finally, tellurium is used to induce hindlimb paralysis in experimental animals via lipofuscinosis (Doull, 1996).

1.10 Theiler's murine encephalomyelitis virus (TMEV)

TMEV infection is a model for the progressive form of MS (Lipton, 1994). TMEV is a positive sense, single-stranded RNA virus in the family *Picornaviridae* (Pevear et al., 1987). TMEV was first characterized as a

murine model for MS in 1975 when it was discovered that TMEV infection may result in a relapsing-remitting demyelination pattern (Lipton, 1975). Vpg is a viral protein necessary for the translation of viral RNA on smooth endoplasmic reticulum. Four capsid proteins, VP1, VP2, VP3, and VP4, are produced which form an icosahedral structure from proteins 1D, 1B, 1C, and 1A respectively. Viral proteins 2B and 2C are needed for viral replication. 3A and 3B are utilized in the initiation of RNA synthesis. 3C is the viral protease that cleaves the genomic polyprotein, and 3D is the viral RNA polymerase which is able to lengthen RNA primers. The L protein has been suspected to play a role in viral pathogenesis due to differences in the L region between TMEV strains. The function of protein 2A and the receptor for TMEV are still unknown (Figure 1.1). TMEV strains have been divided into two subgroups composed of the neurovirulent GDVII and FA substrains and the less virulent TO substrains. The TO substrains include BeAn 8386, DA, WW, TO_4 and Yale (Lipton, 1978, 1980).

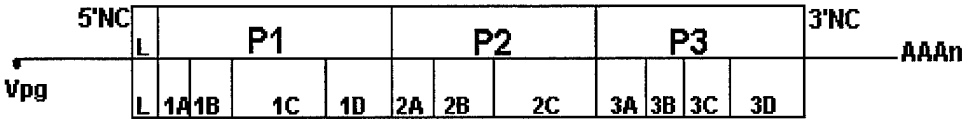

Figure 1.1: TMEV genome.

There are differences in susceptibility to Theiler's virus infection among different inbred strains of mice (Lipton and Dal Canto, 1979).

The highly susceptible strains include SJL/J, DBA/1, DBA/2, SWR/J, NZW, RIIIS/J, and PL/J mice. AKR, CBA, and C3H strains are intermediately susceptible and 129Sv, C57BL/6, C57BL/10, C57L and BALB/c strains are resistant (Nicholson et al., 1994).

1.11 TMEV-induced demyelination (TVID)

Theiler's virus-induced demyelination is a very involved process. Not only is demyelination a direct result of viral replication, but the host immune reaction to viral infection plays a significant role in the development of demyelinating lesions. The exact process of demyelination in TMEV infection is still being debated. Several theories include the activity of viral replication, $CD8^+$ cytotoxic T-cells, $CD4^+$ T-cells, antibody production to virus or myelin, and IFN-γ during the course of infection.

Intracranial (i.c.) inoculation of susceptible mice with neurotropic GDVII results in acute fatal encephalitis during the first week of infection (Dal Canto and Lipton, 1975). Alternatively, permissive hosts infected i.c. with one of the TO substrains of TMEV develop a flaccid paralysis by 2 weeks post-infection (p.i.), by which time viral antigens and genomic RNA are located in the neurons of the gray matter (Dal Canto and Lipton, 1975).

The BeAn strain of TMEV is capable of inducing demyelination in the CNS. The demyelinating phase of TMEV is preceded by a "gray matter" disease (Lipton, 1980). During the first four weeks of infection, virus is found replicating in neurons following intracranial injection of BeAn (Dal Canto and Lipton, 1975). The chronic persistence of TMEV in the white matter of the spinal cord begins 1 month p.i. and continues throughout the duration of infection (Miller and Gerety, 1990, Yamada

et al., 1991). By six weeks post-infection, virus can be detected in astrocytes, macrophages and oligodendrocytes and continues to replicate in these cells until the animal is terminated. Clinical signs of demyelination can be detected as early as 8 weeks post-infection, indicating that the white matter of the spinal cord has begun to be affected.

Persistent viral infection of the CNS is necessary for the development of demyelinating lesions (Bureau et al., 1993; Pritchard et al., 1993). During the course of chronic TMEV infection, Th1 cells specific to TMEV are reported to be the direct cause of a DTH reaction that occurs (Miller and Gerety, 1990). Susceptible strains of mice have a limited T-cell defense and are unable to clear virus efficiently. This lead to the conclusion that viral replication plays a part in the development of demyelinating lesions. One theory is that direct viral lysis of oligodendrocytes can cause demyelination (Rodriguez et al., 1983; Blakemore et al., 1988). Viral infection of these cells can interfere with myelin synthesis and viral lysis can spread infectious virus to astrocytes and macrophages that keep the extracellular space surrounding the oligodendrocytes free of chemical and physical debris (Miller et al., 1997). The release of viable virons may also increase the infection of BeAn in other oligodendrocytes. BeAn has been detected in demyelinating lesions via immunoperoxidase staining and viral particles have been detected in oligodendrocytes by electron microscopy giving merit to the idea that viral replication in oligodendrocytes plays a role in the production of demyelinating lesions (Rodriguez et al., 1983; Brahic et al., 1984; Blakemore et al., 1988; Rossi et al., 1997).

The MHC-class I molecules randomly bind proteins found in the intracellular matrix and express the proteins as peptides on the surface of the cell that the peptides are derived from. MHC-class I molecules are

located on the surface of all nucleated cells including oligodendrocytes. $CD8^+$ cytotoxic T-cells (CTLs) are able to recognize and kill cells that have been infected with TMEV when viral fragments are presented via MHC-class I molecules. When CTLs lyse viral infected oligodendrocytes, demyelination occurs. CTLs may contribute to extracellular viral spreading by lysing viral-infected cells that contain infectious virus (Murray et al., 1998; Rivera-Quinones et al., 1998). The role of $CD8^+$ cells in TVID is controversial, though. Treatment of susceptible mice with anti-$CD8^+$ antibodies does not alter the course of TMEV-induced demyelination (Borrow et al., 1992), and viral infection can result in demyelination even in β_2-microglobulin-deficient resistant mice lacking functional $CD8^+$ T-cells (Pullen and Kim, 1991; Fiette et al., 1993). It has been proposed that a poor $CD8^+$ T-cell response may actually account for the viral persistence in SJL/J mice since low levels of virus-specific CTLs in these mice have been noted (Lindsley et al., 1991; Lin et al., 1998).

MHC-class II molecules are located on the surface of antigen presenting cells (APCs). Dendritic cells are the most efficient APCs though astrocytes and macrophages may also function in this capacity. Peptides that are contained within MHC-class II molecules are presented to $CD4^+$ T-cells. Th1 and Th2 are both subtypes of $CD4^+$ cells though they release different cytokines. IFN-γ is produced by Th1 cells and induces MHC-class II expression on astrocytes (Borrow and Nash, 1992) and cerebrovascular endothelial cells of TMEV susceptible but not TMEV resistant mice (Welsh et al., 1993). The upregulation of MHC-class II results in increased viral or autoantigen presentation which, in turn, increases the production of antibodies to these antigens (Miller et al., 1997). The MHC-class II T-cell lines derived from demyelinating lesions in the spinal cords of SJL/J mice react with the VP1, VP2 and VP3 viral

capsid proteins (Yauch and Kim, 1994; Cameron et al., 2001) and T-cells recognizing VP1 produce the highest levels of IFN-γ. MHC-class II or $CD4^+$ T-cell inactivation/depletion via antibody treatment is able to suppress demyelination caused by TMEV (Rodriguez et al., 1986; Welsh et al., 1987; Yauch et al., 1998) further implicating the role of $CD4^+$ T-cells in TVID. In addition, interfering with the activation of the Th1 cells with cyclosporine treatment reduces the incidence of demyelination with TMEV infection (Rodriguez and Lindsley, 1992).

Inactivation of IL-12, the Th1 cytokine needed for the clonal expansion and priming of T-cells for high IFN-γ production (Manetti et al., 1994), result in an increase in clinical presentation when antibody to IL-12 is given at the time of infection with Theiler's virus. TNF-α and IFN-γ production decrease and IL-10 and IL-4 are increased (Inoue et al., 1998). This correlates with the need for Th1 cytokines during initial viral infection.

In concert with the antibody production, IFN-γ secretion results in Th1 cell proliferation and the suppression of Th2 lymphocytes. Th1 cells are responsible for the DTH reaction to myelin which contributes to demyelination in Theiler's virus infection (Miller et al., 1997; Borrow et al., 1998). Increasing the virus-specific DTH reaction in susceptible mice correlates with the development of increased disease severity (Clatch et al., 1985), which supports a role for Th1-mediated CNS inflammation. Spleen cells from infected mice are able to demyelinate *in vitro* (Dal Canto et al., 2000) and autoimmune responses to epitopes of myelin membrane have been detected which suggests T-cells are produced against myelin as a result of infection (Miller et al., 1997).

Another consequence of MHC-class II restricted DTH $CD4^+$ T-cell activation is the recruitment of macrophages which release proteases, reactive oxidative radicals, and TNF-α which contribute to bystander

demyelination (Vanguri and Shin, 1988). Therefore, bystander demyelination is not a direct effect of $CD4^+$ T-cells, but rather the activation of macrophages. Macrophages secrete proteases as waste into the extracellular environment. When macrophages are activated to remove myelin debris via endocytosis in an area of demyelination, eventually, the macrophage will become gorged with debris. The proteases that are produced while the macrophage undergoes cytoplasmic digestion of the debris are able to degrade myelin which subsequently damage oligodendrocytes and myelin.

1.12 MHC haplotypes and TMEV

In TVID, inflammation and MHC haplotypes play an important role in susceptibility. Mice with heterozygous or homozygous dominant MHC class I *H2-D* allele *b*, are resistant to TVID (Azoulay-Cayla et al., 2000). Animals with *d*, *k*, and *s* haplotypes are intermediately susceptible to TMEV, yet mice with *p*, *q*, *r* and *f H2-D* alleles develop chronic disease when infected with TMEV (Clatch et al., 1985, 1987; Rodriguez and David, 1985; Rodriguez et al., 1986, 1990; Patick et al., 1990; Brahic and Bureau, 1998). The resistant *b* haplotype is dominant (Bureau et al., 1992). Two other genes, *Tmevp2* and *Tmevp3* have been implicated in the persistence of TMEV in susceptible mice through the use of multiple background crosses. The exact function of these genes is still unknown (Bihl et al., 1999).

One of the major differences between the three groups of inbred mice stated above is that susceptible mice produce less IFN-γ post-infection and fewer cytotoxic T-cells. These mice are unable to clear the virus efficiently before it infects oligodendrocytes (Chamorro et al., 1986; Lipton et al., 1991, 1998). The oligodendrocytes are lysed by CTLs

resulting in macrophage infiltration and demyelination. Susceptible mice also have a higher DTH response to myelin late in the disease process that contributes to inflammation and demyelination (Borrow et al., 1998).

1.13 Theiler's virus and interferons

IFN-α/β receptor knockout mice have demonstrated the importance of type I interferons in TVID. 129Sv mice were utilized in this study. The capacity to respond to endogenous IFN-α/β is necessary for survival of 129Sv mice to the chronic phase of TMEV. Heterozygous or homozygous dominant *H-2D^b* haplotype 129Sv mice are naturally resistant to the DA strain of Theiler's virus whereas 129Sv mice that are homozygous recessive for *H-2D^b* are susceptible to persistent infection (Azoulay-Cayla et al., 2000).

Infection with the DA Theiler's strain produces a similar disease as BeAn infection in susceptible mice. 129Sv or BALB/c mice that lack the IFN-α/β receptors die of severe encephalitis due to DA infection and those that lack IFN-γ receptors develop extreme late inflammatory and demyelinating disease. High viral load of DA was observed throughout the CNS tissue (Fiette, et al. 1995; Van den Broek et al., 1995; Njenga et al., 1997). Conversely, SJL/J mice infected with DA and treated with mouse IFN-α/β for five weeks experienced remyelination and a decrease in clinical presentation of disease. However, long-term treatment resulted in an exacerbation of disease (Njenga et al., 2000). This work re-enforces the importance of interferon activation in resistance to initial viral infection and the potentially dangerous long-term effects IFN-β treatment may pose on MS patients.

1.14 DTH and Theiler's virus

Type IV delayed-type hypersensitivity is a form of cell-mediated immunity that occurs 24-48 hours after challenge with an antigen previously encountered by the host. This is a T-cell mediated response resulting in the infiltration of macrophages and inflammation. In the case of a Theiler's virus infection, the major protein of concern is host myelin basic protein when the focus is autoimmunity. Myelin that was prepared from the brains of adult SJL mice initiates a DTH reaction within 24 hours when injected into the ears of SJL mice chronically infected with TMEV strain BeAn. The intensity of the DTH reaction is assessed via measurement of ear thickness where PBS injections into the opposite ears of the same mice are used as controls (Borrow et al., 1998; Slavin et al., 1998; Wang et al., 1999).

During the course of chronic TMEV infection, Th1 cells specific to TMEV are reported to be the direct cause of a delayed-type hypersensitivity reaction that occurs and contributes to bystander demyelination (Miller and Gerety, 1990; Borrow et al., 1998). DTH responses may also be used to determine the immune reactivity to TMEV using UV-irradiated whole virus particles (Pullen et al., 1994; Lipton et al., 1995; Drescher et al., 1998; Murray et al., 1998; Koh et al., 2000), active TMEV (Karpus et al., 1995; Inoue et al., 1996; Inoue et al., 1997; Inoue et al., 1998; Inoue et al., 1999), or specific viral peptides (Miller et al., 1995; Burt et al., 1999; Pavelko et al., 2000). In all of these experiments, the DTH response was used as an assessment of the efficiency of the immune system to recognize virus after either an immunomodulating treatment or a genetic mutation that affects MHC presentation.

Disease susceptibility in TVID correlates with the temporal development of chronic high levels of TMEV-specific, MHC-class II-restricted DTH responses, but not with virus-specific serum antibody responses or with viral titers in the CNS (Gerety et al., 1991; Gerety et al., 1994). DTH responses to PLP, MOG, (Miller et al., 1997) and whole myelin (Borrow et al., 1998) have been reported during the late demyelinating phase of TMEV in SJL/J mice. The phenomenon of epitope spreading occurs during TVID, whereby mice develop segmental T-cell responses to different myelin epitopes during the course of the disease (Miller et al., 1997). Furthermore, these autoreactive T-cells appear to contribute to the demyelinating process since spleen cells from infected mice are able to demyelinate *in vitro* cultures following stimulation with PLP (Dal Canto et al., 2000). Thus, DTH T-cells reacting to both virus and myelin appear to play a role in inflammatory demyelination in this model.

1.15 Experimental allergic encephalomyelitis (EAE)

EAE focuses on the autoimmune component of MS and is induced by the injection of myelin basic protein (Meyer et al., 1996), whole spinal cord homogenate (Brown et al., 1981) or proteolipid protein plus adjuvant into laboratory animals including mice (Trotter et al., 1987; Fritz and McFarlin, 1989; Ke and Kapp, 1996). EAE can also develop through adoptive transfer of $CD4^+$ T-cells to naïve mice which establishes it as an autoimmune model for MS (Pettinelli and McFarlin, 1981; McCarron and McFarlin, 1988; Satoh et al., 1988; Fallis et al., 1989; Kuchroo et al., 1992).

The development of clinical symptoms begins 6-8 days after adoptive transfer occurs and is characterized as changes in gait, hind

limb paralysis, and incontinence. These symptoms last for about two weeks after which the animal may recover slightly. Other relapses will continue to occur and will become progressively worse over time (Dal Canto et al., 1995).

EAE mimics MS in that it is an autoimmune inflammatory disease of the CNS that is T-cell-mediated (Zamvil and Steinman, 1990). The upregulation of IL-2, IFN-γ, and TNF-β (Van der Veen and Stohlman, 1993; Cua et al., 1995; Olsson, 1995) and the down regulation of TGF-β, IL-10, and IL-4 (Okuda et al., 1995; Issazadeh et al., 1996; Diab et al., 1997) have been detected in the CNS of animals with EAE resulting in the conclusion that development of disease is the result of Th1 cell cytokine secretion and the balance between Th1/Th2 cell activation (Van der Veen and Stohlman, 1993; Cua et al., 1995; Olsson, 1995). Chronic-relapsing EAE results in inflammatory cell activation in the CNS and is seen in the meninges, perivascular spaces, and eventually the parenchyma. Axonal destruction is less extensive in EAE than in Theiler's virus, though macrophages remain in the CNS tissue for many weeks in both models. In chronic-relapsing EAE, the amount of inflammation seen in the CNS is not constant. During the remitting stages of disease, inflammation is reduced as it is in RR MS (Dal Canto et al., 1995).

1.16 Sex differences in TMEV and EAE

Susceptibility to Theiler's virus and EAE vary in different sexes of inbred strains of mice. Male and female SJL/J mice are both equally susceptible to TMEV infection with BeAn 8386 (Kappel et al., 1990) though male SJL/J mice are less susceptible to the TMEV DA strain than female SJL/J mice (Hill et al., 1998). EAE is induced to a greater

degree in female as opposed to male SJL/J mice (Voskuhl et al., 1996). For the extent of this work, female SJL/J mice were used exclusively so that comparisons could be made to previous reports of IFN-τ treated, female SJL/J mice with chronic EAE (Soos et al., 1995; Majtaba et al., 1997; Soos et al., 1997; Majtaba et al, 1998).

1.17 Cytokine expression in the CNS during chronic EAE or TMEV

The expression of Th1 and Th2 cytokines in the CNS during EAE or TMEV infection is important to note since it is believed the change in cytokine expression is partially responsible for the induction of demyelination seen in both animal models. During the exacerbation phase of EAE in SJL/J mice, 14 days post-inoculation, Th1 cytokines IL-2 and IFN-γ are up-regulated in the brain, spinal cord, and cerebrospinal fluid (Renno et al., 1994).

The cytokine data concerning chronic TMEV infection is controversial. Using the DA strain of TMEV, at 40 days p.i. in SJL/J mice, Sato et al. found that the Th1 cytokines IL-1 and IL-2 were not detectable in the spinal cord whereas Th2 cytokines, IL-10, and Th1 cytokines TNF-α, IL-12, and IFN-γ were elevated when compared to controls. In the brains of the same animals, IL-10, IL-12, TNF-α, IL-1, IL-2, IL-6, and IFN-γ were not detected at 40 days p.i. but, IL-4 production was high in the brain (Sato et al., 1997). It has also been reported that IFN-γ, IL-1, IL-2, IL-6, IL-12, TNF-α, TGF-β1, IL-4, IL-5 and IL-10 cytokine RNA was detected at levels above controls at 60 days p.i. in SJL/J mice also infected with DA (Chang et al., 2000). High levels of lymphotoxin-β, TNF-α, IL-6, and TGF-β were observed in the brains of SJL/J mice infected with DA for 60 days in a separate study (Theil et al.,

2000). Since there appears to be inconsistencies between 40 and 60 days p.i. with the same TMEV strain, it would be interesting to see if there was a difference in cytokine expression in the CNS of mice infected with the BeAn as compared to the DA strain of TMEV.

1.18 Interferon-tau in sheep

IFN-τ has been characterized as one of the pregnancy recognition signals that is secreted by sheep trophoblasts between days 9 and 24 of pregnancy (Godkin et al., 1984). IFN-τ has 70% homology with IFN-α (Stewart et al., 1989), and is therefore classified as a type I interferon. Only trophoblast cells and their derivatives are able to transcribe IFN-τ due to the promoter region –126 to –450 base pairs from the transcriptional start site. This unique promoter region accounts for the majority of the 30% sequence difference between IFN-τ and the other type I interferons. It enables the expression of IFN-τ to be dependent upon pregnancy (Cross and Roberts, 1991). IFN-τ functions as an anti-luteolytic agent by decreasing the endometrial gene expression of oxytocin receptors (Vallet and Lemming, 1991; Spencer et al., 1996). Though a threshold for IFN-τ effectiveness may be dose dependent since it has been reported that high doses of IFN-τ may result in apoptosis in sheep hepatocytes *in vivo* (Kim et al., 2000).

1.19 Immunomodulator functions of IFN-τ

Unlike the other type I interferons, IFN-τ is not induced by viral infection and double stranded RNA (Roberts et al., 1992). It's synthesis is maintained over a period of days as opposed to hours as is the case with IFN-α and IFN-β (Godkin et al., 1982). IFN-τ acts in an anti-inflammatory capacity *in vivo* by inhibiting Th1 lymphocyte proliferation (Newton et al., 1989, Niwano et al., 1989; Skopets et al., 1992; Assal-Meliani et al., 1993), promoting the production of placental Th2 cytokines IL-4 and IL-10 (Chaouat et al., 1995), and activating NK cells *in utero* (Tuo et al, 1993).

1.20 The anti-viral effects of IFN-τ

The anti-viral activity of IFN-τ is comparable or superior to IFN-α in ovine lentivirus, human papilloma virus, feline immunodeficiency virus, and human immunodeficiency virus infections without the cytotoxicity seen with IFN-α at high concentrations.

Ovine lentivirus titers are reduced by 90% as compared to controls in lambs that receive IFN-τ during the first month of infection. Inflammation is also reduced in the lungs of treated lambs (Juste et al., 2000). IFN-τ decreases the intracellular levels of E6 and E7 of human papilloma virus in normal human keratinocytes, which induces apoptosis via increasing p53 concentrations (Johnson et al., 1999). Feline lymphocytes infected with FIV and human lymphocytes infected with HIV-1 do not reduce in viability as a result of IFN-τ treatment (Pontzer et al., 1997). In a similar study, IFN-τ had a 35-fold antiviral effect over IFN-α in peripheral blood lymphocytes infected with HIV. In

monocyte-derived macrophages, IFN-τ is 100 fold more efficient in inhibiting HIV replication than IFN-α. Both IFN-α and IFN-β reduced cell viability at 50,000 U/ml whereas IFN-τ did not (Dereuddre-Bosquet et al., 1996). Finally, IFN-τ is able to inhibit TMEV replication, up regulate MHC class I and down regulate MHC class II in cerebral vascular endothelial cells (Tennakoon et al., 2001).

1.21 Interferon treatment of EAE

EAE has been used as a model to understand the mechanism of action of the beneficial affects of IFN-β seen in MS since this is poorly understood. Recombinant mouse IFN-β is able to successfully treat chronic EAE in SJL/J and (SWR x SJL)F_1 mice via reduction of IFN-γ, increase in IL-10 and TGF-β production, and inhibition of DTH response (Brod and Burns, 1994; Yu et al., 1996; Yasuda et al., 1999).

IFN-τ has proven to be an effective treatment in EAE by preventing acute induction and superantigen reactivation of EAE via $CD4^+$ T-suppressor cells that inhibit MBP activation of T-cells (Mujtaba et al., 1997). This protection occurs without the toxicity that is seen with high concentrations of IFN-α and IFN-β treatment (Soos et al., 1995). Oral as well as i.p. administration of IFN-τ in EAE give the same amount of protection, but oral administration does not result in the production of non-neutralizing antibodies to IFN-τ that is seen with i.p. delivery. The current hypothesis is that this protection occurs via Th2 cytokine activation. The Th2 proliferation theory is supported by an increased IL-10 production during IFN-τ treatment, the inhibition of B-cell and T-cell responses in active and chronic EAE *in vivo* and *in vitro*, and the lack of

inflammatory lymphocytic lesions and activation of microglia in the CNS during IFN-τ treatment (Soos et al., 1997; Majtaba et al, 1998).

1.22 Hypothesis

Dual roles exist for the T-cells involved in Theiler's virus-induced demyelination. T-cells are required for the decrease in viral load during the early course of infection, but are known to play a part in the demyelination that occurs during chronic infection. Both $CD4^+$ and $CD8^+$ cells are important in viral clearance during acute disease. Mice depleted of $CD4^+$ cells do not survive the initial viral infection (Welsh et al., 1987) and mice depleted of $CD8^+$ T-cells suffer high viral titers and subsequently more severe demyelinating disease (Borrow et al., 1992). However, the severity of demyelination and inflammation in chronic TMEV can be reduced with immunosuppressive therapies such as anti-CD4 antibody treatment (Welsh et al., 1987) and cyclophosphamide administration (Lipton and Dal Canto, 1976).

Based on the aforementioned anti-viral and cytokine-modulating effects of IFN-τ *in vivo* and during EAE, as well as the immunosuppressive data on TMEV, it is believed there is a possibility that IFN-τ treatment may reduce the development of and/or degree of demyelinating lesions in the CNS of chronically TMEV-infected mice. Different time points for treatment during the course of infection need to be investigated in order to assess the appropriate time frame where immunosuppression with IFN-τ would be beneficial. After the development of a persistent TMEV infection in the CNS, IFN-τ treatment should aid in the upregulation of Th2 cytokines thereby decreasing Th1 proliferation, which may result in a decrease in disease progression. Alternatively, IFN-τ treatment administered before viral infection is

expected to result in immunosuppression and increase initial viral load thereby enhancing lesion development.

Specific Aims

1. To determine the most effective time course of IFN-τ treatment in SJL/J mice during different stages of disease resulting from TMEV infection and to compare IFN-τ treatment with treatment using a known anti-inflammatory, methylprednisolone. Weekly clinical scoring and weights will be used to determine disease progression.

2. To analyze histological changes in the spinal cords of mice treated with IFN-τ in the areas of demyelination, status spongiosus, and inflammation as opposed to infected non-treated and treated non-infected controls. H+E and slides stained for CNS myelin will be used.

3. To investigate the mechanism of action of IFN-τ treatment as it relates to immunosuppression. Serum levels of IL-10 and corticosterone will be measured by ELISA and RIA analysis respectively. Circulating antibody levels to IFN-τ will also be measured via ELISA. DTH to myelin will be measured as an indication of Th1 cell activation. Th1 and Th2 cytokine expression in the spinal cord of 4-6 month IFN-τ treated group will be documented through the use of RNase protection assay (RPA) analysis.

II. CLINICAL DISEASE PROGRESSION

Introduction

2.1 Interferon treatment in multiple sclerosis

Interferon-beta (IFN-β) has a therapeutic effect in MS patients by reducing the severity of MS symptoms and relapse rate (Paty et al., 1993; Jacobs et al., 1996). However, several undesirable side effects have been noted. Fever, myalgia, headache, fatigue, and chills occur in 75% of treated patients though the duration of these side effects is patient specific (Lublin et al., 1996). This may be due to the immediate upregulation of inflammatory cytokines and prostaglandins (Dayal et al., 1995; Brod et al., 1996; Arnason, et al., 1994) resulting in increased spasticity and temporary worsening of symptoms (Lublin et al., 1996).

Occasionally, lymphopenia, neutropenia, leukopenia, and increased liver aminotransferase values are detected in MS patients as a result of IFN-β administration (PRISMS study group, 1998). Pre-menopausal women may experience breakthrough bleeding or spotting during IFN-β treatment due to a decrease in serum progesterone and estradiol levels (Vial et al., 1994; Kauppila et al., 1982).

Changes in mood, specifically pessimism, depression, and lack of interest in treatment can be increased in MS patients undergoing IFN-β treatment, though this may be a side-effect of chronic disease (Mohr et al., 1996; Mohr et al., 1997; Borras et al., 1999). Reaction at the site of injection is dependent upon the method of administration with subcutaneous administration resulting in hardened skin lesions, uninflammed sclerotic dermal plaques, erythematous plaques, ulcers,

and sarcoid-like granulomatous dermatitis (Elgart et al., 1997; Mehta et al., 1998).

2.2 Interferons in Theiler's virus infected mice

Interferon-alpha (IFN-α) and IFN-β are both classified as type I interferons. The capacity to respond to endogenous IFN-α/β is necessary for survival of 129Sv mice following infection with TMEV (Azoulay-Cayla et al., 2000). 129Sv knockout mice that lack the IFN-α/β receptors die of severe encephalitis due to DA infection and those that lack IFN-γ receptors develop extreme late inflammatory and demyelinating disease as well as a high viral load of DA throughout the CNS tissue (Fiette, et al. 1995). BALB/c knockout mice without the IFN-α/β receptor also develop severe encephalitis and expire within a two day period when infected with DA (Njenga et al., 1997). This work re-enforces the importance of interferon activation in resistance to initial viral infection.

Administration of type I interferons to TMEV infected mice has beneficial effects during a short term treatment but detrimental effects during a long term treatment. Five week administration of biologically effective doses of IFN-α/β increased remyelination significantly. A threefold increase in oligodendrocyte remyelination was reported though the extent of demyelination in the spinal cord was not statistically significant between the five-week treated and control groups. Sixteen-week treatment with IFN-α/β increased the absolute area of remyelination in the CNS, but the demyelinating lesions were more extensive and numerous to the extent that total remyelination was negligible. Based on *in situ* hybridization, it was noted that CNS remyelination during the five-week treatment was independent of

replicating virus yet the extent of demyelination in the spinal cord directly correlated to the amount of virus positive cells. On the other hand, there was no correlation found in the sixteen-week treated group between the extent of demyelination and viral load, suggesting the two types of demyelination occur via separate mechanisms (Njenga et al., 2000).

2.3 Interferon-tau

Since it is known that IFN-α/β can be both beneficial in short term treatments yet harmful in long term treatments in TMEV, another type I interferon, IFN-τ, has been investigated in this study. IFN-τ has 70% homology with IFN-α (Stewart et al., 1989), and is therefore classified as a type I interferon with anti-viral activity comparable or superior to IFN-α in the treatment of ovine lentivirus *in vivo* and *in vitro* (Juste et al., 2000), human papilloma virus *in vitro* (Johnson et al., 1999), and HIV *in vitro* (Dereuddre-Bosquet et al., 1996). In common with the other type I interferons, IFN-α and IFN-β, IFN-τ inhibits T-cell proliferation in response to superantigen stimulation in human peripheral mononuclear cells with staphylococcal enterotoxin B, staphylococcal enterotoxin A, and toxic shock syndrome toxin 1. In contrast to IFN-α/β, IFN-τ does not induce cytotoxicity at concentrations greater than 10^4 units/mL (Soos and Johnson, 1995).

IFN-τ has been characterized as one of the pregnancy recognition signals that is secreted by sheep trophoblasts between days 9 and 24 of pregnancy (Godkin et al., 1984). It functions as an anti-luteolytic agent by decreasing the endometrial gene expression of oxytocin receptors (Vallet and Lemming, 1991; Spencer et al., 1996).

IFN-τ also acts in an anti-inflammatory capacity *in vivo* by inhibiting Th1 lymphocyte proliferation (Newton et al., 1989; Niwano et al., 1989; Skopets et al., 1992; Assal-Meliani et al., 1993), promoting the production of placental Th2 cytokines IL-4 and IL-10 (Chaouat et al., 1995), and activating NK cells *in utero* (Tuo et al, 1993).

2.4 Interferon-tau and EAE

EAE, experimental allergic encephalomyelitis, another model of MS, is induced by the injection of myelin basic protein (Meyer et al., 1996) or proteolipid protein plus adjuvant into laboratory animals including mice (Ke and Kapp, 1996). EAE can also develop through adoptive transfer to naïve mice which establishes it as an autoimmune model for MS (McCarron and McFarlin, 1988; Satoh et al., 1988; Kuchroo et al., 1992).

IFN-τ has proven to be an effective treatment in EAE by preventing acute induction and superantigen reactivation of EAE via induction of suppressor cells (Soos et al., 1995; Mujtaba et al., 1997). In addition, IFN-τ administration initiates remission in SJL/J mice with chronic EAE and prevents subsequent relapses (Mujtaba et al., 1998) as well as preventing the acute and chronic forms of EAE when administered orally (Soos et al., 1997). The current hypothesis is that protection occurs via Th2 cytokine activation as noted with increased IL-10 and TGF-β production (Soos et al., 1997), the inhibition of B-cell and T-cell responses in active and chronic EAE (Mujtaba et al, 1998), and the lack of inflammatory lymphocytic lesions in the CNS (Soos et al., 1997) during IFN-τ treatment.

2.5 Methylprednisolone

Another anti-inflammatory agent, methylprednisolone, was utilized in the present study. Methylprednisolone is known to improve recovery of CNS function in human patients with acute damage to the spinal cord (Bracken et al., 1990) and greatly reduces inflammation in rats after partial transection of the spinal cord (Bartholdi and Schwab, 1995). In a double-blind trial where MS patients were examined for 1 year, intravenous infusion of methylprednisolone in saline was found to be as effective in promoting MS remission as dexamethasone (LaMantia et al., 1994). Furthermore, methylprednisolone administration to SJL/J mice following lysolecithin-induced demyelination, initiates remyelination. A reduction in the total number of macrophages found in the spinal cord lesions was also noted suggesting a decrease in the inflammatory response is beneficial to remyelination (Pavelko et al., 1998).

2.6 Cytokine expression in TMEV

Immunosuppressive treatment of TMEV has been favorable when treatments focus on decreasing inflammatory responses during late disease. The use of cyclophosphamide or monoclonal antibodies specific for $CD4^+$ or $CD8^+$ T-cells result in a decrease in demyelination when administered during chronic TMEV infection (Rodriguez and Lindsley, 1992). In contrast, immunosuppression with cyclophosphamide that began during early disease, five-weeks post-infection, does not affect demyelination when compared to controls (Roos et al., 1982) possibly due to the need for Th1 cytokines to control early viral titers.

Regulating the type of T-cell response by the use of severe compromised immunodeficent (SCID) mice or the administration of anti-

$CD4^+$ T-cell and/or anti-$CD8^+$ T-cell antibodies have been helpful in determining the role of these cells in disease progression. The depletion of $CD4^+$ T-cells in mice prior to TMEV infection results in high mortality rates 3-5 weeks after infection and lack of antiviral IgG response. Adoptive transfer of $CD4^+$ T-cells restore survival rates to normal suggesting that $CD4^+$ T-cells are protective after viral load has decreased (Borrow et al., 1993).

Adoptive transfer of $CD8^+$ T-cells confers resistance to TMEV-induced demyelinating disease in susceptible recipient mice (Nicholson et al., 1996) supporting theory that $CD8^+$ CTLs are protective after disease onset. SJL/J and CBA mice depleted of $CD8^+$ T-cells prior to infection with TMEV suffer from a more severe demyelinating disease than do infected control mice (Borrow et al., 1992) suggesting that $CD8^+$ T-cells are also important in the reduction of viral load during initial infection. Recently, it was reported by Haynes et al. that $CD8^+$ cells that are removed from BeAn infected, resistant BALB/cByJ mice are able to specifically down regulate IFN-γ and IL-2 production and decrease the DTH Th1 reaction when given to BALB/cByJ animals that have been irradiated and therefore become susceptible to BeAn via regulatory $CD8^+$ T-cell depletion (Haynes et al., 2000).

The regulation or inactivation of cytokines directly is also used as a type of immunosuppression. Administration of antibody to IFN-γ, which is normally a natural anti-viral agent, before TMEV infection of SJL/J mice, results in an increase in disease progression and severity. Mortality rate is not increased due to IFN-γ antibody administration (Rodriguez et al., 1995). In contrast, an overwhelming fatal CNS TMEV infection is seen with cyclophosphamide immunosuppressed (Lipton et al., 1976; Rodriguez et al., 1991), T-cell-depleted athymic nude (Rosenthal et al., 1986) or neonatal (Rodriguez et al., 1983) mice.

Suppression of TMEV-induced demyelinating disease onset is seen with pentoxifylline administration due to the depletion of Th1 cytokines TNF-α and IFN-γ and the upregulation of Th2 cytokines IL-4 and IL-10 when SJL/J mice infected with BeAn are treated –2 to 12 days post-infection (Fushimi et al., 1998). Though this data is contradictory to the IFN-γ data reported above, there is a possibility this discrepancy is due to the fact that the Fushimi group infected mice at 6 weeks of age whereas normally SJL/J mice are infected with TMEV between 3-5 weeks of age when the CNS is not fully myelinated.

The regulation of the immune response is vital to the outcome of TMEV infection. As stated above, depletion of the $CD4^+$ T-cells that produce Th1 cytokines early or before TMEV infection results in an increase in viral load and mortality in susceptible mice. $CD8^+$ T-cell depletion prior to infection also results in increased viral load. In contrast, during chronic disease, suppression of both $CD4^+$ and $CD8^+$ T-cells appear to lead to decreased inflammatory demyelinating disease. We propose that the immunomodulating effects of interferon-tau may play a significant role in remyelination during chronic TMEV infection whereas early administration may not beneficial due to the down regulation of Th1 cytokine production.

Materials and Methods

2.7 Mice

3-week-old female SJL/J mice (Harlan, Houston, TX) were obtained and infected when 4-5 weeks old.

2.8 Virus

The BeAn 8386 strain of TMEV was a gift from Dr. Howard L. Lipton (Northwestern University, Evanston, IL). Virus was grown in BHK-21 cells, and the culture supernatant containing infectious virus was aliquoted and stored at -70°C before use. The viral titer was determined by plaque assay on BHK-21 cells (Rueckert & Pallansch, 1981).

2.9 Interferon-tau

The IFN-τ was a gift from Dr. Fuller W. Bazer (Texas A&M Health Science Center, College Station, TX). IFN-τ was isolated as previously described (Ott et al., 1991), diluted in 20mM Tris buffer pH 7.5 with 150mM NaCl, aliquoted and stored at -70°C before use. Protein concentration was determined by the Lowry assay (Lowry et al., 1951).

2.10 Infection of mice and assessment of clinical signs of demyelination

Mice were anesthetized with Metofane™ (Schering-Plough Animal Health, Union, NJ) and injected intracranially (i.c.) into the right cerebral hemisphere with 5 x 10^4 plaque forming units (PFU) of BeAn in a 20 uL volume.

After infection (day 0) all animals were examined weekly for the development of clinical signs indicative of demyelinating disease (Borrow et al., 1998). Clinical signs were scored on a scale from 0 to 6, 0 indicating a healthy animal and 1-6 representing gradual increasing severity of signs as follows: 1, piloerection and/or hunched posture; 2, piloerection and hunched posture plus unsteady gait; 3, very unsteady gait, weak grasp response when placed on wire grid, and occasional slight limb monoparesis; 4, severe hind limb weakness, incontinence, and weight loss; 5, paraparesis of hind limbs, severe weight loss, incontinence, dropped head; 6, moribund/dead.

2.11 IFN-τ treatment of mice prior to Theiler's virus infection up to the development of moderate demyelinating disease

Ten mice were injected with 10^5 units of IFN-τ intraperitoneally daily from -3 to 0 days post-infection and 10 mice were treated with the same concentration of IFN-tau –3 to 60 days post-infection. Ten virus-infected control mice for each treatment received 100uL of 0.9% saline i.p. during the same intervals. All perfusions occurred at 6 months post-infection.

2.12 IFN-τ treatment of mice with moderate demyelinating disease

Ten mice were injected with 10^5 units of IFN-τ i.p. daily from 2 months to 6 months post-infection. Ten virus infected control mice received 100uL of 0.9% saline i.p. at the same intervals. All perfusions occurred at 6 months post-infection.

2.13 IFN-τ treatment of mice with severe demyelinating disease

A replication of the same experiment was performed to test the therapeutic potential of IFN-τ in mice suffering from severe demyelinating disease. In each experiment, 10 mice were injected with 10^5 units of IFN-τ (Soos et al., 1995) i.p. daily from 4 months to 6 months post-infection. Ten virus-infected control mice received 100uL of 0.9% saline i.p. at the same intervals. All perfusions occurred at 6 months post-infection.

2.14 Methylprednisolone treatment of mice

Ten mice were injected i.p. with pulse injections of 1mg methylprednisolone (40mg/kg) (Pharmacia & Upjohn, Kalamazoo, MI) every three days from 5 months to 6 months p.i. to inhibit inflammatory responses (Pavelko et al., 1998). Ten virus infected control mice received 100uL of 0.9% saline i.p. at the same intervals. All perfusions occurred at 6 months post-infection.

2.15 IFN-τ treatment of non-infected control mice

Ten mice were treated from 5 to 7 months of age daily with 10^5 units of interferon-tau by i.p. injection. Ten non-infected control mice were injected with 100uL of 0.9% saline i.p. at the same time. The mice were perfused at 7 months of age.

2.16 Neurological assessment

Neurological exams of the –3 to 60 day post-infection mice were performed at days 0, 90, and 170 post-infection. The mice were evaluated by Dr. Joan Coates (Department of Small Animal Medicine and Surgery, Texas A&M College of Veterinary Medicine) using a previously published protocol for rat neurological and behavioral analysis (Whishaw, et al., 1983) that had been modified by Dr. Coates for mice (see appendix for score sheet). Dr. Coates evaluated all of the mice without prior knowledge of the treatment groups.

2.17 Statistical analysis

A repeated measures analysis of covariance (ANCOVA) was performed on the weights and clinical scores of the subjects over time. When appropriate, pre-treatment weights or clinical scores served as a covariate.

Results

2.18 IFN-τ treatment of mice prior to Theiler's virus infection up to the development of moderate demyelinating disease

All treatment groups are summarized in table 2.1. Treatment of the infected SJL/J mice with IFN-τ three days before infection to the day of infection resulted in a significant weight loss, $p<0.003$, beginning at 18 weeks p.i. (figure 2.1) and an increase in clinical disease onset, $p<0.001$, from 7 weeks p.i. which continued throughout infection (figure 2.2). Treatment with IFN-τ three days before infection to sixty days post-infection showed the same pattern of disease development with significant weight loss, $p<0.0002$, at 18 weeks p.i. (figure 2.3) and worsening of clinical symptoms, $p<0.0001$, (figure 2.4) at 7 weeks p.i. as a result of IFN-τ treatment. According to the neurological assessment, both the pretreated groups developed a significant increase in neurological impairment with stereotyped behaviors including a significant decline in locomotor behavior $p<0.004$, a decline in gait ataxia which approached significance $p<0.06$, increased resting tremor occurrence which approached significance $p<0.06$, and increase aggravated cage behavior which also approached statistical significance $p<0.06$ when compared to saline treated, virus infected control animals.

Table 2.1. Experimental groups in IFN-τ treatment study using SJL/J female mice and TMEV.

Infected	Treatment	Time Course Treated	n
+	IFN-tau	-3 to 0 days pi	10
+	Saline	-3 to 0 days pi	10
+	IFN-tau	-3 to 60 days pi	10
+	Saline	-3 to 60 days pi	10
+	IFN-tau	2 to 6 mo. pi	10
+	Saline	2 to 6 mo. pi	10
+	IFN-tau	4 to 6 mo. pi	20
+	Saline	4 to 6 mo. pi	20
-	IFN-tau	5-7 mo.	10
-	Saline	5-7 mo.	10
+	Methylprednisolone	5-6 mo. pi	10
+	Saline	5-6 mo. pi	10
-	None	N/A	6

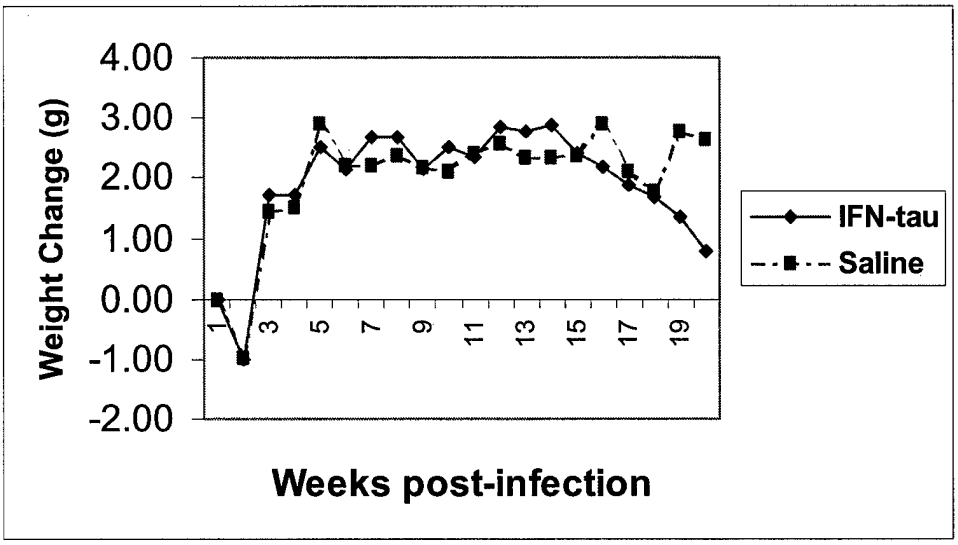

Figure 2.1: Mean weight change IFN-τ treatment –3 to 0 days post-infection. Change from baseline of 10 BeAn infected female SJL/J mice treated with IFN-τ -3 to 0 days post-infection and 10 BeAn infected saline controls.

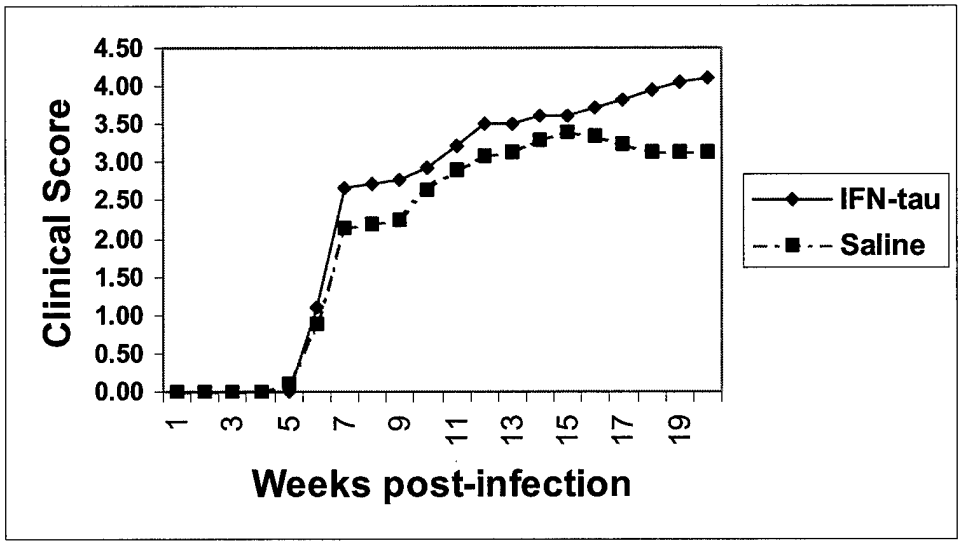

Figure 2.2: Mean score change IFN-τ treatment –3 to 0 days post-infection. Mean clinical scores from baseline of 10 BeAn infected female SJL/J mice treated with IFN-τ –3 to 0 days post-infection and 10 BeAn infected saline controls.

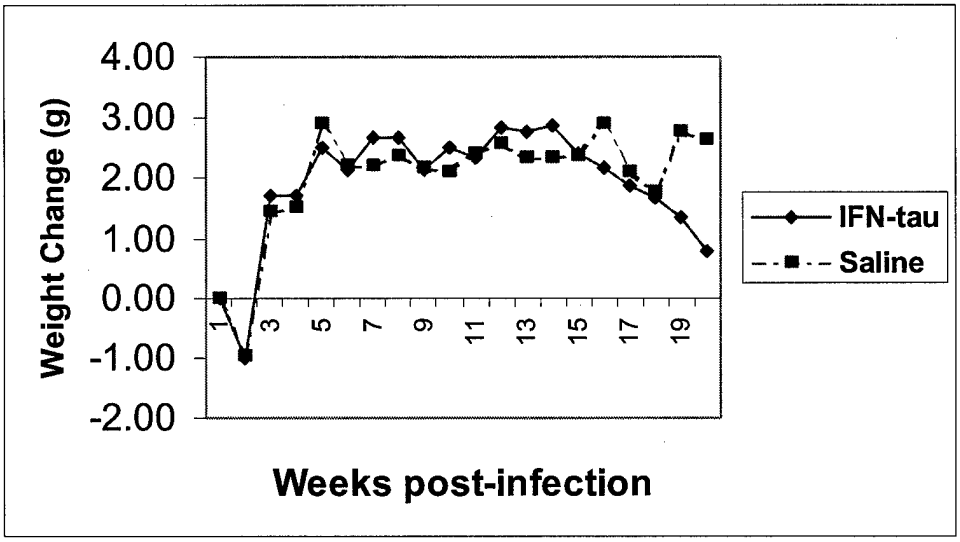

Figure 2.3: Mean weight change IFN-τ treatment –3 to 60 days post-infection. Mean weight change from baseline of 10 BeAn infected female SJL/J mice treated with IFN-τ -3 to 60 days post-infection and 10 BeAn infected saline controls.

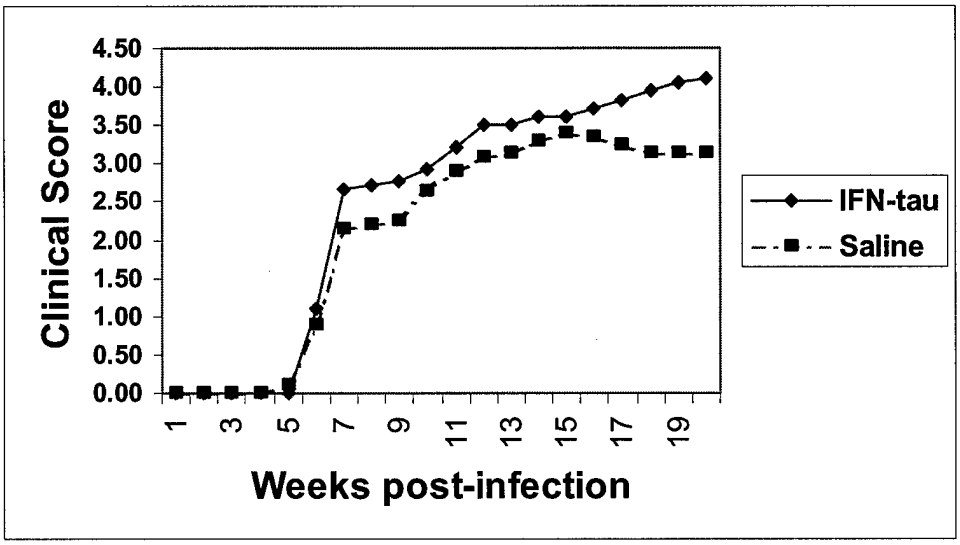

Figure 2.4: Mean score change IFN-τ treatment –3 to 60 days post-infection. Mean clinical scores of 10 BeAn infected female SJL/J mice treated with IFN-τ –3 to 60 days post-infection and 10 BeAn infected saline controls.

2.19 IFN-τ treatment of mice with moderate demyelinating disease

Interferon-tau treatment from two-months post-infection to six-months post-infection did not change the course of disease progression as compared to the saline controls in regards to weight gain, $p<0.2312$, (figure 2.5). However, at 11-weeks post-treatment, a divergence is seen where the IFN-τ treated mice begin to gain weight (figure 2.5). This separation has not been shown to be significant but is worth further evaluation. Clinically, these mice did improve slightly after 8-weeks of treatment and continued to do so until the end of the study, $p<0.07$, (figure 2.6).

2.20 IFN-τ treatment of mice with severe demyelinating disease

During the chronic phase of disease caused by TMEV, a significant improvement in weight gain was noted beginning week 5 of treatment with a slight divergence at week 6 (figure 2.7), $p<0.0001$, which was consistently repeated (figure 2.11), $p<0.0079$, and clinical scores decreased (figure 2.8) with significant differences between groups occurring at 4-weeks post-treatment, $p<0.0001$, (figure 2.12), $p<0.0053$. These effects were observed when IFN-τ was administered during the four to six months post-infection time frame. Figures 2.9 and 2.10 indicate the changes in clinical score immediately prior to IFN-τ treatment and then after two months of treatment. With the exception of one mouse, all of the mice improved clinically (figure 2.9). The one outlier was noted in this group which had severe clinical symptoms at the beginning of the treatment (figure 2.9). There appears to be a

threshold based on the severity of disease before treatment where IFN-τ administration is not beneficial to the animal. Disease progression continued in the saline treated, virus infected control animals (figure 2.10).

2.21 Methylprednisolone treatment of mice

The animals treated with methylprednisolone every third day displayed some overall weight gain that was marginally significant after day 5 of treatment, $p<0.0697$, (figure 2.13) and decreased in clinical scores, $p<0.0756$, (figure 2.14) as compared to virus infected, saline controls, but the animals continued to show marked signs of continuing demyelinating disease progression.

2.22 IFN-τ treatment of non-infected control mice

Non-infected mice suffered marginally significant weight loss which began after three weeks of daily treatment and continued throughout the treatment period due to IFN-τ administration, $p=0.065$, whereas the non-infected, saline control mice had no weight loss (figure 2.15).

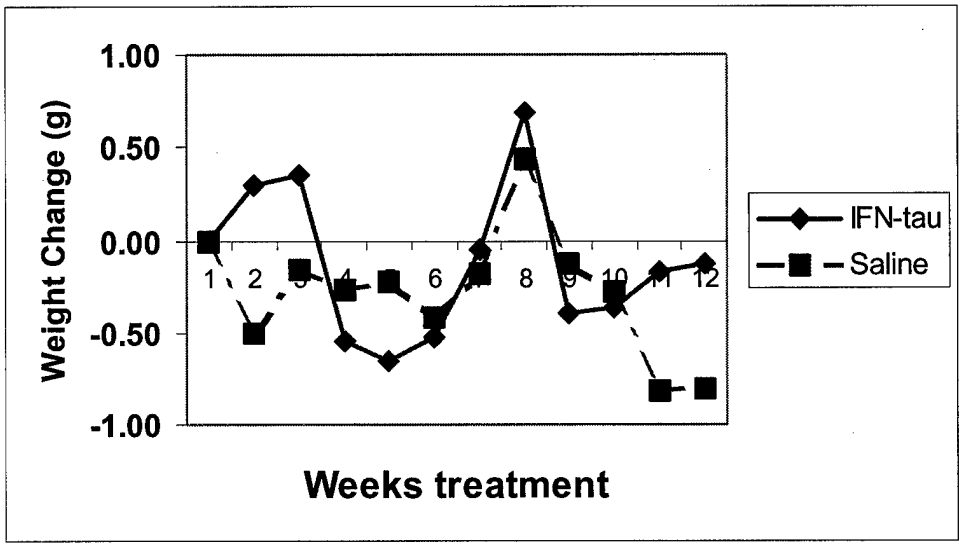

Figure 2.5: Mean weight change IFN-τ treatment 2 to 6 months post-infection. Mean weight change from baseline of 10 BeAn infected female SJL/J mice treated with IFN-τ 2 to 6 months post-infection and 10 BeAn infected saline controls.

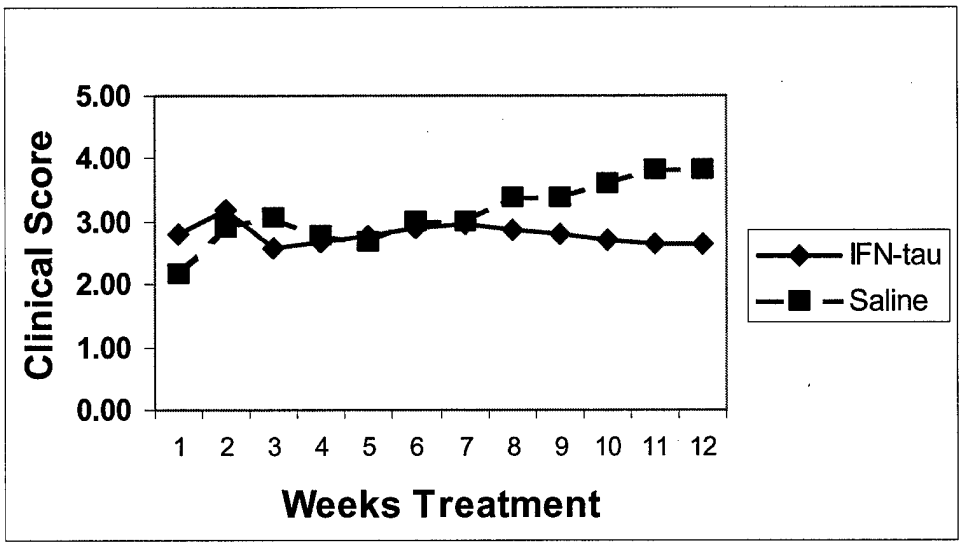

Figure 2.6: Mean score change IFN-τ treatment 2 to 6 months post-infection. Mean clinical scores of 10 BeAn infected female SJL/J mice treated with IFN-τ 2 to 6 months post-infection and 10 BeAn infected saline controls.

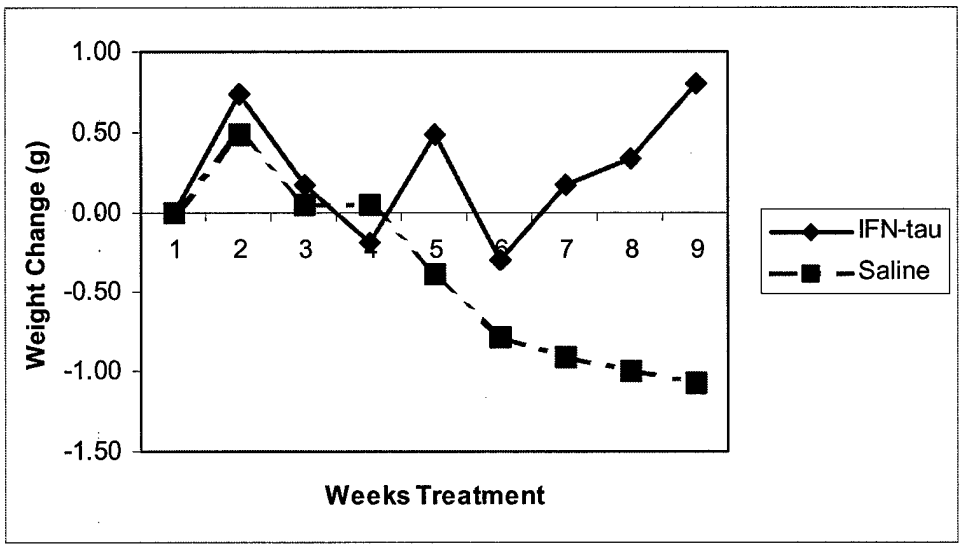

Figure 2.7: Mean weight change IFN-τ treatment 4 to 6 months post-infection. Mean weight change from baseline of 10 BeAn infected female SJL/J mice treated with IFN-τ 4 to 6 months post-infection and 10 BeAn infected saline controls.

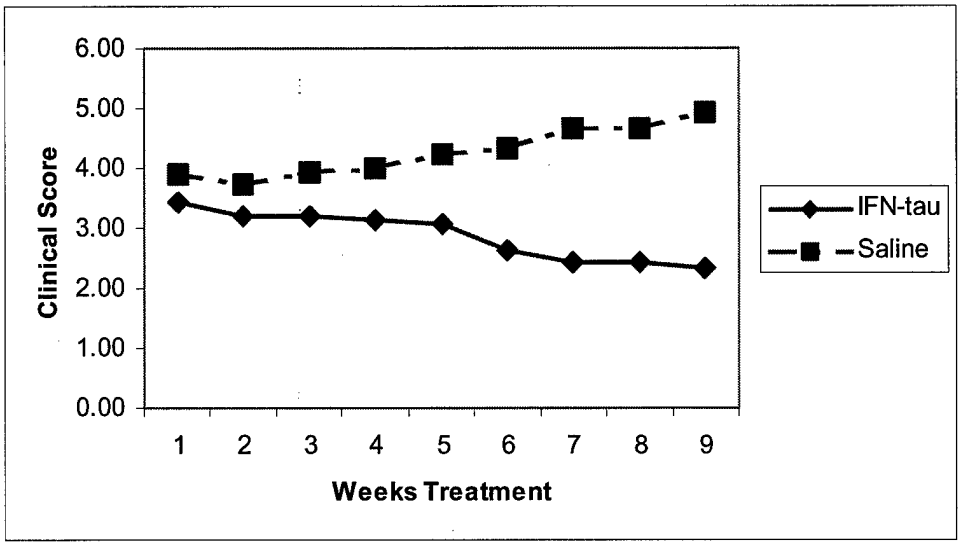

Figure 2.8: Mean score change IFN-τ treatment 4 to 6 months post-infection. Mean clinical scores of 10 BeAn infected female SJL/J mice treated with IFN-τ 4 to 6 months post-infection and 10 BeAn infected saline controls.

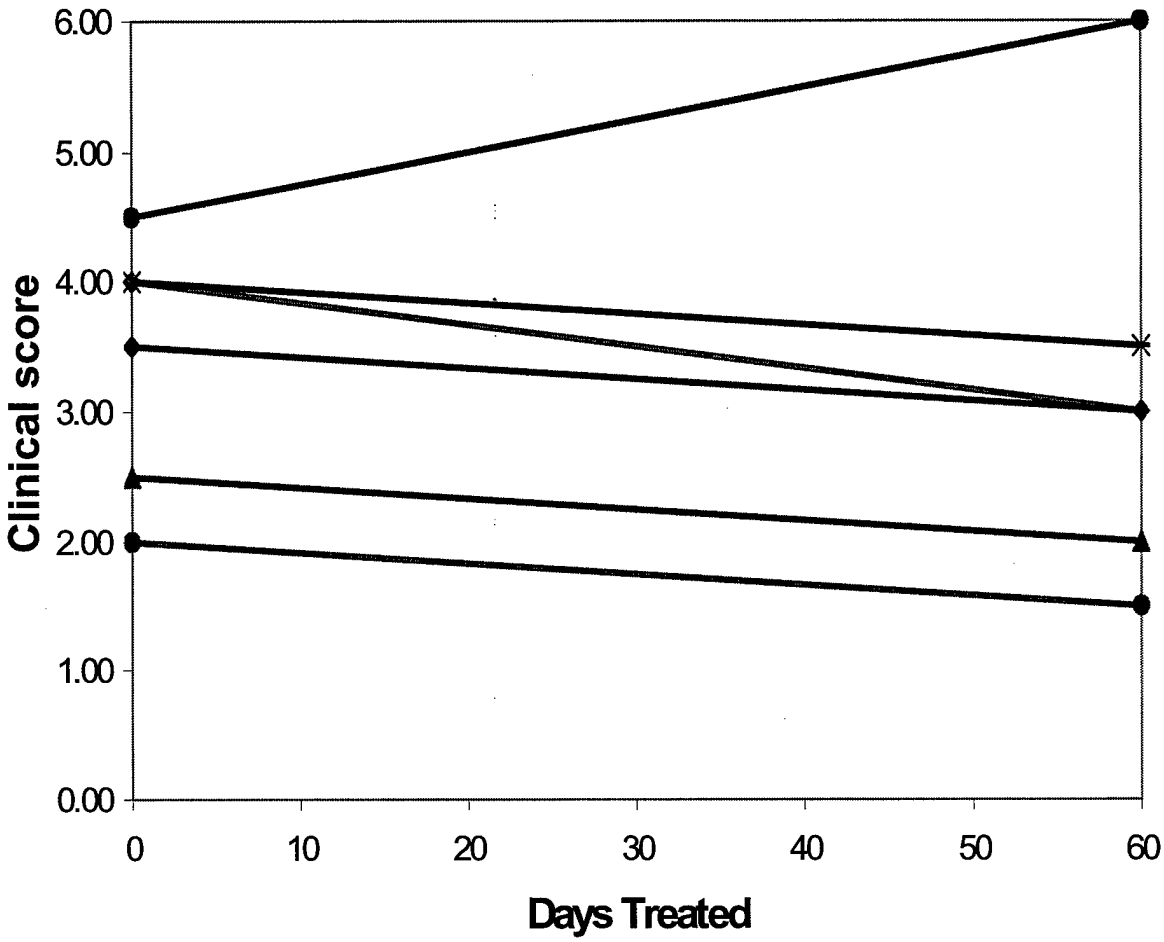

Figure 2.9: Individual score change before and after IFN-τ treatment 4 to 6 months post-infection.

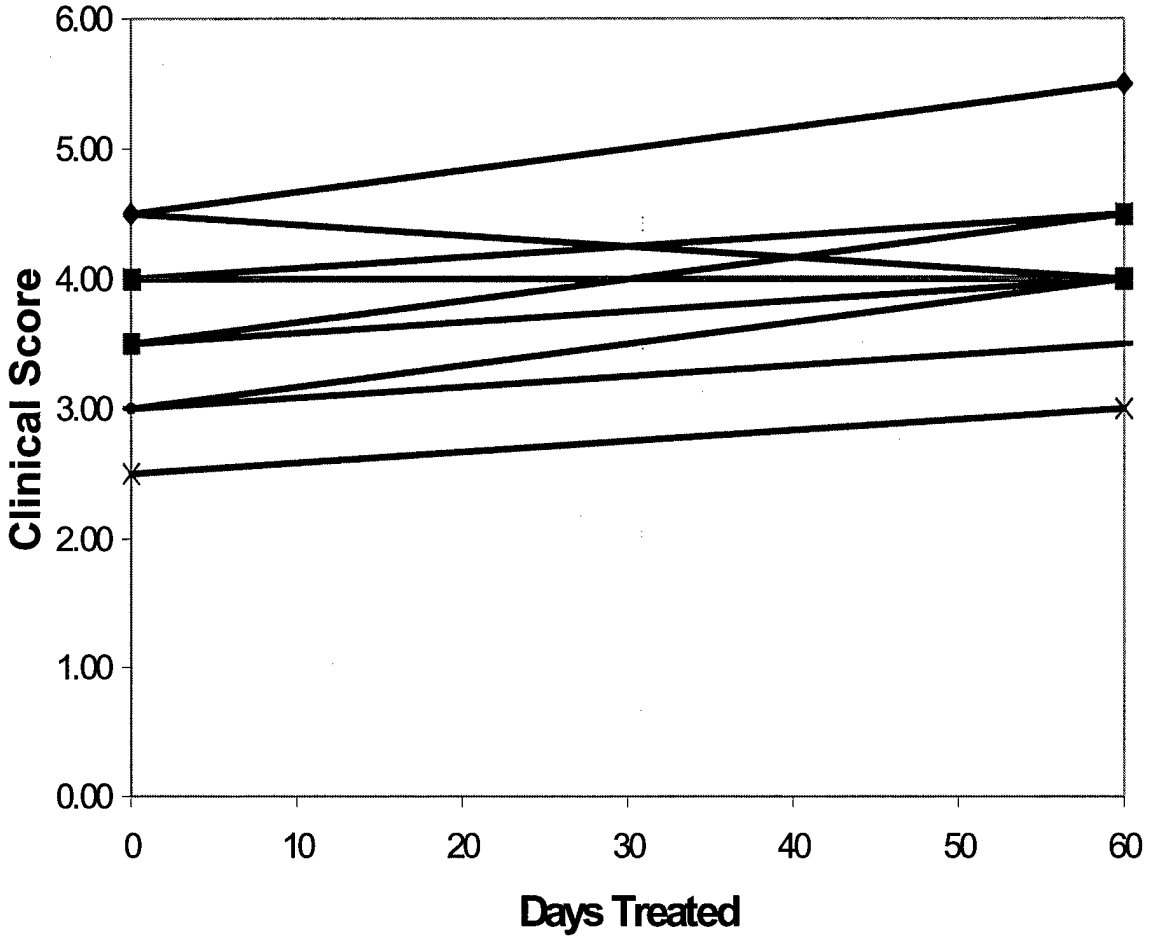

Figure 2.10: Individual score change before and after saline treatment 4 to 6 months post-infection.

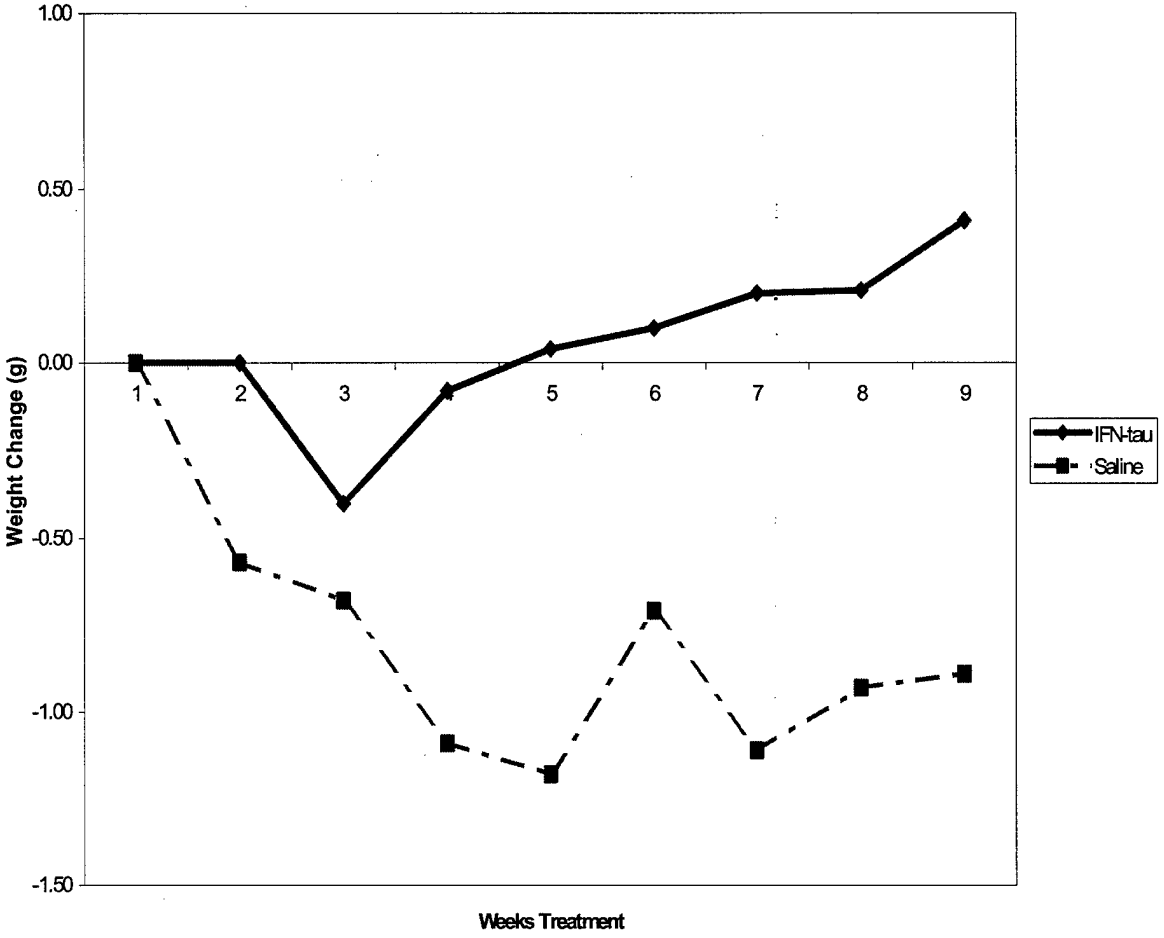

Figure 2.11: Mean weight change IFN-τ treatment 4 to 6 months post-infection #2. Mean weight change from baseline of all 10 BeAn infected female SJL/J mice treated with IFN-τ 4 to 6 months post-infection and 10 BeAn infected saline controls for the same period.

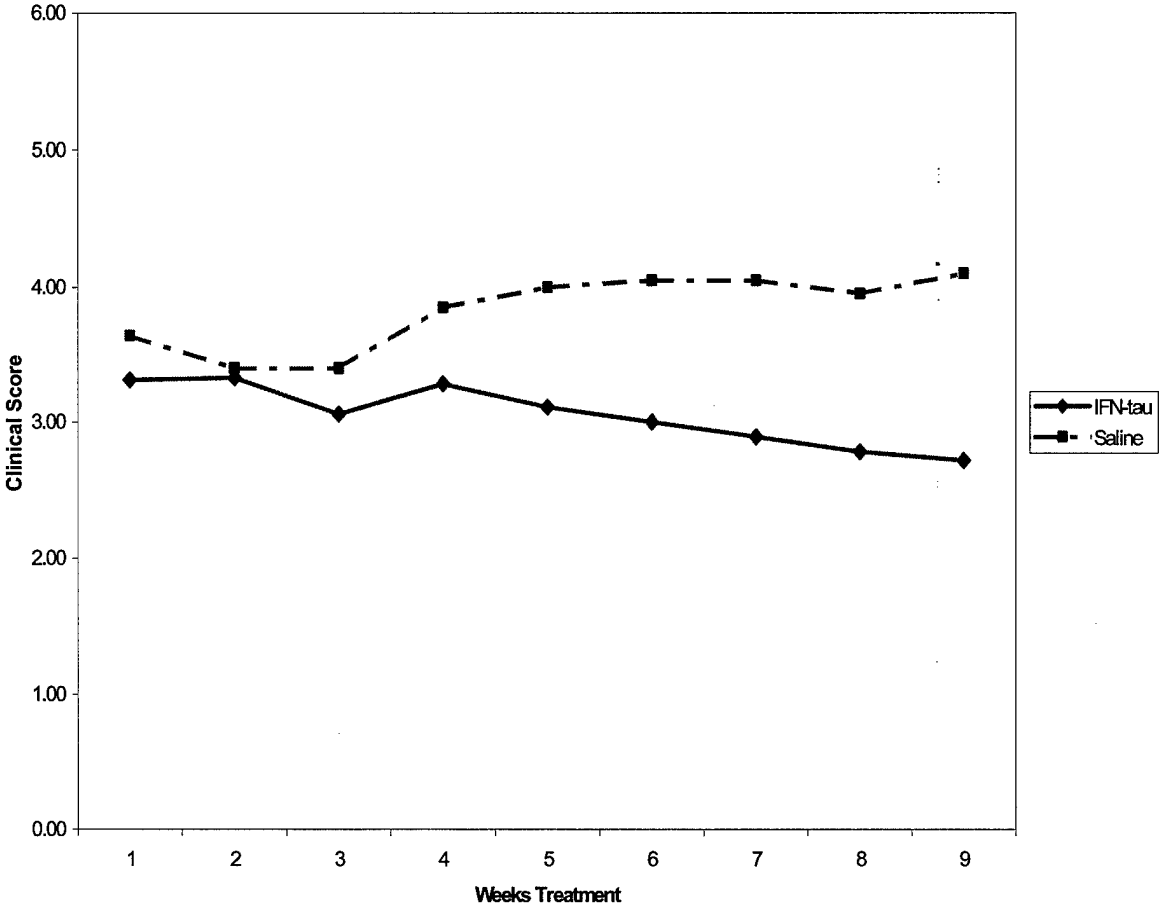

Figure 2.12: Mean score change IFN-τ treatment 4 to 6 months post-infection #2. Mean clinical scores of 10 BeAn infected female SJL/J mice treated with IFN-τ 4 to 6 months post-infection and 10 BeAn infected saline controls.

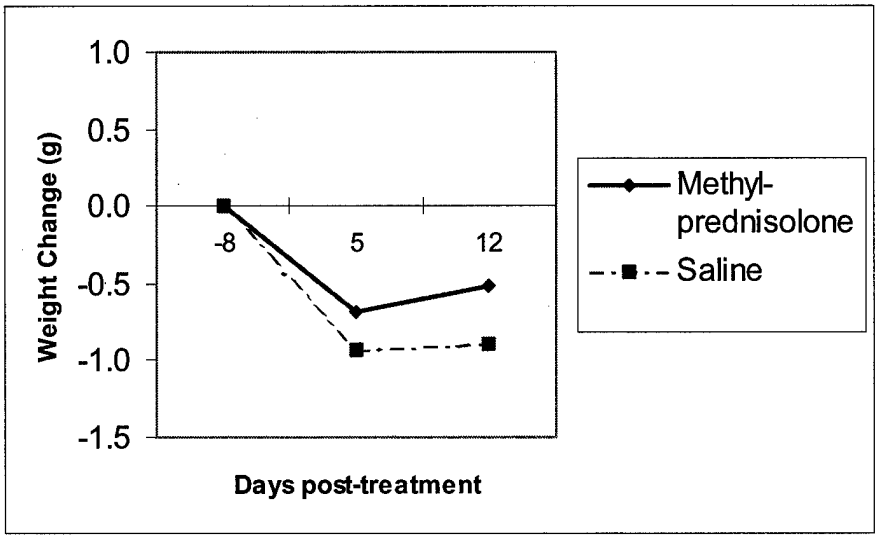

Figure 2.13: Mean weight change methylprednisolone 5 to 6 months post-infection. Change from baseline of 10 BeAn infected female SJL/J mice treated with methylprednisolone from 5 to 6 months post-infection every 3 days and 10 BeAn infected saline controls.

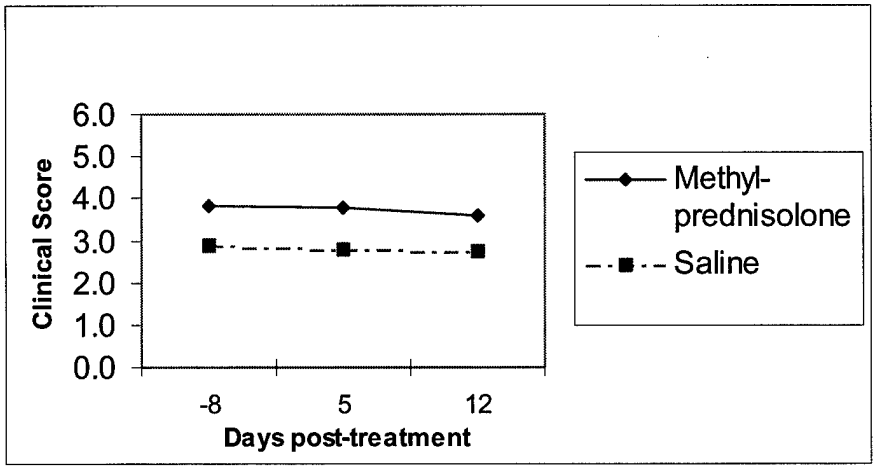

Figure 2.14: Mean score change methylprednisolone 5 to 6 months post-infection. Clinical scores of 10 BeAn infected female SJL/J mice treated with methylprednisolone from 5 to 6 months post-infection every 3 days and 10 BeAn infected saline controls.

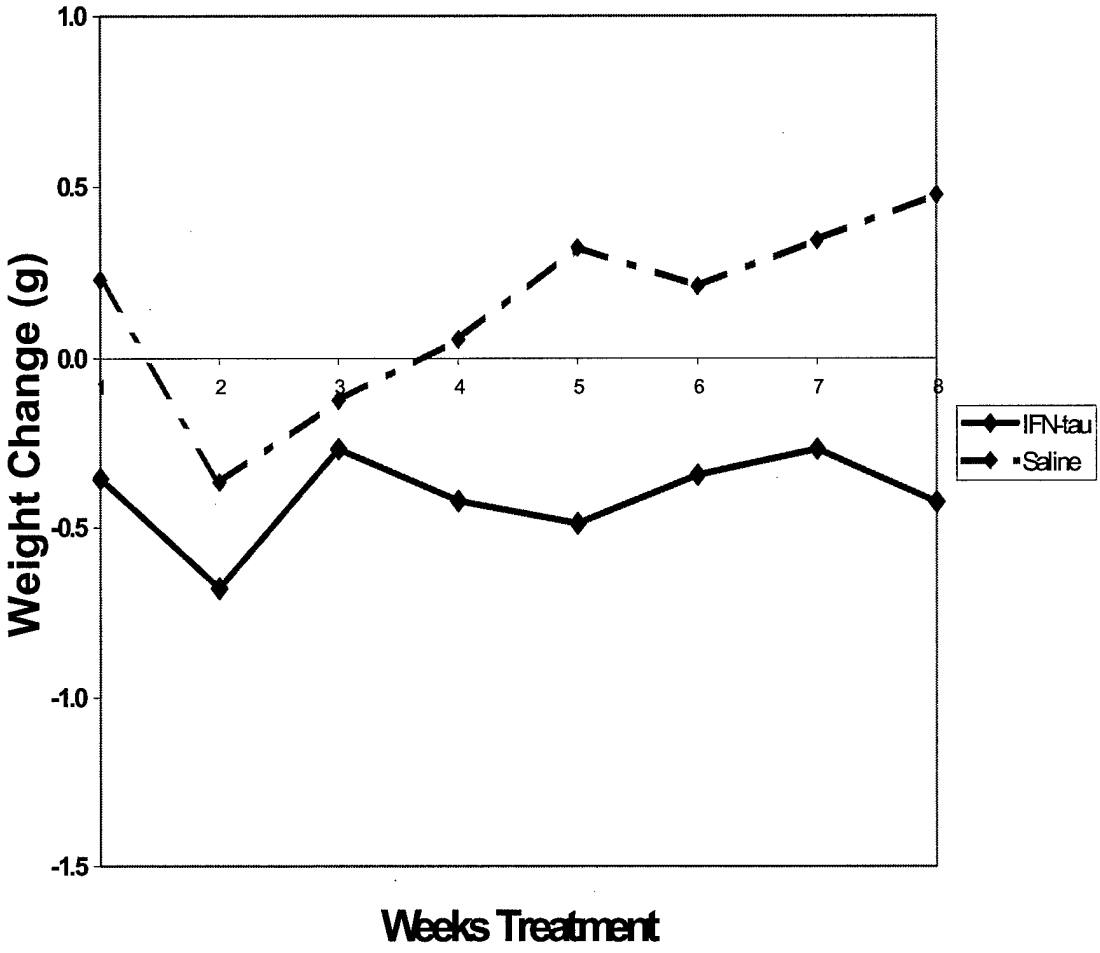

Figure 2.15: Mean weight change of non-infected mice with IFN-τ treatment 5 to 7 months of age. Change from baseline of 10 non-infected female SJL/J mice treated with IFN-τ 5 to 7 months of age and 10 non-infected saline controls of the same age.

Discussion

IFN-τ has been shown to prevent the development of the acute form of EAE in NZW mice and the development of chronic-relapsing EAE in SJL/j mice (Soos et al., 1995). In addition to protecting against both acute and chronic forms of EAE, parental or oral administration of IFN-τ has been shown to alleviate chronic-relapsing EAE in SJL/j mice (Soos et al., 1997). The effectiveness of IFN-τ in the treatment of active EAE and its ability to exert an effect when administered orally without the induction of neutralizing antibodies makes IFN-τ a more attractive therapeutic agent for the treatment of MS.

Since MS is thought to be initiated by a viral infection, the effectiveness of type I interferons may be related to their role in viral inhibition. In addition, the autoimmune aspects of MS may be down regulated through a number of different mechanisms since type I IFNs have been shown to inhibit DTH responses (also important in TVID and EAE), alter the homing and trapping of lymphocytes, enhance NK and T-cell activity and cytotoxicity, enhance Fcγ receptors effects (Belardelli and Gresser, 1996), inhibit T-cell proliferation, and decrease the production of IFN-γ (Noronha et al., 1993).

The administration of type I interferons, IFN-α/β, in SJL/J mice infected with the DA strain of Theiler's virus results in increased demyelination when give 4.5-months p.i for sixteen weeks (Njenga et al., 2000). IFN-α/β given short term results in the promotion of remyelination. The authors noted that the mice lost weight during weeks 6-7 of treatment, developed reduced DTH responses to TMEV and had decreased numbers of T-cells and B-cells infiltrating the CNS. The treated mice developed antibodies to IFN-α/β which did not appear to

affect the efficacy of the treatment. Furthermore, IFN-α/β treatment did not affect viral replication as measured by *in situ* hybridization quantification of RNA-positive cells. Therefore, the therapeutic effect was not mediated by the antiviral effects of IFN-α/β, but rather by the immunomodulatory functions.

The current study indicates that IFN-τ, also a type I interferon, is an effective therapeutic agent for the treatment of mice with the late stages of TVID, but administration during early disease progression can exacerbate clinical presentation. Mice that were given IFN-τ before viral infection were significantly worse than control mice, possibly as a result of the suppression of the Th1 cytokine response during initial infection (Borrow et al., 1993). There was only a slight significant difference noted between the 2-6 month p.i. treated mice and the infected controls at the end of treatment. It is possible continued exposure to IFN-τ may be of additional benefit to these mice, but additional experiments are required to address this issue.

The 4-6 month post-infection treated mice were the most affected by IFN-τ. They gained weight and improved clinically, similar to the mice that were $CD4^+$ T–cell deficient late in disease progression (Nicholson et al., 1996). Mice were treated with IFN-τ at four months post infection in an attempt to mimic the situation in MS where the mean time for diagnosis is two years at which time lesions are well established.

When non-infected mice were dosed with IFN-τ, a marginally significant weight loss was detected three weeks after administration when compared to saline controls. This confirms that the statistically significant weight gain seen in the 4-6 month p.i. treated group was a real effect of treatment. It is interesting to note, however that IFN-τ may

have limitations in aiding in disease recovery and that the degree of clinical severity at the time of treatment may also be an important factor. One mouse that was clinically scored above 4.0 before treatment did not survive the length of the study. This implies that clinical presentation before treatment may be as important as timing of dosage.

III. HISTOLOGICAL ANALYSIS

Introduction

3.1 Multiple sclerosis

The etiology of MS is unknown, but individual variation in lesions that develop, as well as multiple immunogenetic factors, have raised the possibility that MS may be the result of several different disease processes (Ebers et al., 1996; Haines, et al., 1996; Sawcer et al., 1996). In addition, MS lesions can differ considerably between patients depending on the disease form that is diagnosed. A recent report by Lucchinetti et al. (2000) suggests two different patterns of lesion development, one that is T-cell-mediated or T-cell plus antibody-mediated autoimmune encephalomyelitis and the other involving primary oligodendrocyte damage as the result of a viral or bacterial infection. Of the 83 patients examined, all were found to have both types of MS lesions though multiple active lesions within the same patient were found to involve only one type. The presence of two types of different lesion patterns support a multifactorial etiology for MS.

Cytokine expression also plays a role in the development of demyelinating lesions in MS patients. In order for the MS lesions to occur, it is proposed that myelin self-reacting $CD4^+$ T helper 1 (Th1) cells that can be activated to release proinflammatory Th1 cytokines IL-2, IFN-γ and TNF-β (Bottomly, 1988), must travel from the circulatory system into the CNS (Martin and McFarland, 1995; Lucchinetti and Rodriguez, 1997). T-cells collected from MS patients during relapsing episodes are known to produce the Th1 cytokines: IFN-γ, TNF-α, and TNF-β, (Correale et al., 1995; Voskuhl et al., 1993; Olsson et al., 1990).

Conversely, the Th2 response involves the secretion of anti-inflammatory cytokines such as IL-4 and IL-10 that are prevalent in MS patients during remission phases (Correale et al., 1995). By the secretion of IFN-γ, Th1 cells aid in the switch in immunoglobulin production from IgM to IgG2a as well as inhibit Th2 lymphocyte production. In comparison, Th2 cells facilitate the IgG1 switch to IgE and indirectly suppress Th1 lymphocytes by affecting antigen presenting cells via IL-4, IL-5, and IL-6 (Finkelman, et al., 1990; Mosmann and Coffman, 1989). The possibility that Th1 cytokines may increase the severity of MS lesions is further supported by observation that IFN-γ administration to MS patients results in the worsening of symptoms (Panitch et al., 1987).

3.2 EAE and TMEV lesion comparison

EAE mimics MS in that it is an autoimmune inflammatory disease of the CNS that is T cell-mediated (Zamvil and Steinman, 1990). The upregulation of IL-2, IFN-γ, and TNF-β (Van der Veen and Stohlman, 1993; Cua et al., 1995; Olsson, 1995) and the down regulation of TGF-β, IL-10, and IL-4 (Okuda et al., 1995; Issazadeh et al., 1996; Diab et al., 1997) have been detected in the CNS of animals with EAE resulting in the conclusion that development of disease is the result of Th1 cell cytokine secretion and the balance between Th1/Th2 cell activation (Van der Veen and Stohlman, 1993; Cua et al., 1995; Olsson, 1995). Theiler's virus-induced demyelination (TVID) is also a T-cell dependent demyelination model. This is supported by the failure of adult thymectomized, irradiated, bone marrow-reconstituted susceptible SJL/J mice to develop clinical or microscopic evidence of TVID after i.c. infection with BeAn (Gerety et al., 1994).

The pathological lesions seen in chronic EAE in SJL/J mice are similar to that reported in SJL/J mice infected with BeAn after twelve-weeks post-infection with BeAn. Inflammatory demyelinating lesions are seen in the anterior and lateral funiculi of both EAE and TMEV mice. Inflammatory T-cells accumulate in the subarachnoid space and extend into the spinal cord by traveling through perivascular spaces. Axonal destruction also occurs and is extensive. Meningeal inflammation is prevalent and large amounts of myelin debris can be found in the macrophages of both disease models.

In contrast, SJL/J mice with EAE develop more of a reactive gliosis than mice with TMEV, even though macrophage infiltrates and demyelination are present. The lesion presentation in EAE mirrors TMEV in that the anterior funiculi, lateral funiculi, perivascular spaces, and meninges are the greatest affected. In chronic TMEV infections, new lesions continue to appear throughout disease whereas in chronic relapsing EAE there are times where demyelination is decreased (Dal Canto et al., 1995).

IFN-τ has proven to be an effective treatment in EAE by preventing acute induction and superantigen reactivation of EAE via suppressor cells (Soos et al., 1995; Majtaba et al., 1997). The current hypothesis is that this protection occurs via Th2 cytokine activation as noted with increased IL-10 production (Soos et al., 1997), the inhibition of B-cell and T-cell responses in active and chronic EAE (Majtaba et al, 1998), and the lack of inflammatory lymphocytic lesions in the CNS (Soos et al., 1997) during IFN-τ treatment.

This study focuses on a histological examination of the spinal cords of TMEV-infected mice that were treated with IFN-τ at multiple time points and control animals that were injected with saline during the same treatment periods. Specifically, hematoxylin and eosin

staining, electron microscopy, and immunostaining for CNS myelin was used to analyze the degree of status spongiosus, demyelination, and inflammation within the spinal cord.

Materials and Methods

3.3 Mice

Three-week old female SJL/j mice (Harlan, Houston, TX) were obtained and infected when 4-5 weeks old.

3.4 Virus

The BeAn 8386 strain of TMEV was a gift from Dr. Howard L. Lipton (Northwestern University, Evanston, IL). Virus was grown in BHK-21 cells, and the culture supernatant containing infectious virus was aliquoted and stored at -70°C before use. The viral titer was determined by plaque assay on BHK-21 cells (Rueckert & Pallansch, 1981).

3.5 Interferon-tau

The IFN-τ was a gift from Dr. Fuller W. Bazer (Texas A&M Health Science Center, College Station, TX). IFN-τ was isolated as previously described (Ott et al., 1991), diluted in 20mM Tris buffer pH 7.5 with 150mM NaCl, aliquoted and stored at -70°C before use. Protein concentration was determined by the Lowry assay (Lowry et al., 1951).

3.6 Infection of mice

Mice were anesthetized with Metofane™ (Schering-Plough Animal Health, Union, NJ) and injected intracranially (i.c.) into the right cerebral hemisphere with 5 x 10^4 plaque forming units (PFU) of BeAn in a 20 uL volume.

3.7 IFN-τ treatment of mice prior to Theiler's virus infection up to the development of moderate demyelinating disease

Ten mice were injected with 10^5 units of IFN-τ intraperitoneally daily from 0 to 3 days pre-infection and 10 mice were treated with the same concentration of IFN-tau –3 to 60 days post-infection. Ten infected control mice for each treatment received 100uL of 0.9% saline i.p. during the same intervals. All perfusions occurred at 6 months post-infection.

3.8 IFN-τ treatment of mice with moderate demyelinating disease

Ten mice were injected with 10^5 units of IFN-τ i.p. daily from 2 months to 6 months post-infection. Ten infected control mice received 100uL of 0.9% saline i.p. at the same intervals. All perfusions occurred at 6 months post-infection.

3.9 IFN-τ treatment of mice with severe demyelinating disease

Two experiments were performed to test the therapeutic potential of IFN-τ in mice suffering from severe demyelinating disease. In each experiment, 10 mice were injected with 10^5 units of IFN-τ (Soos et al., 1995) i.p. daily from 4 months to 6 months post-infection. Ten infected control mice received 100uL of 0.9% saline i.p. at the same intervals. All perfusions occurred at 6 months post-infection.

3.10 Methylprednisolone treatment of mice

Ten mice were injected i.p. with pulse injections of 1mg methylprednisolone (40mg/kg) (Pharmacia & Upjohn, Kalamazoo, MI) every three days from 5 months to 6 months p.i. to inhibit inflammatory responses (Pavelko et al., 1998). Ten infected control mice received 100uL of 0.9% saline i.p. at the same intervals. All perfusions occurred at 6 months p.i.

3.11 IFN-τ treatment of non-infected control mice

Ten mice were treated from 5 to 7 months of age daily with 10^5 units of interferon-tau by i.p. injection. Ten non-infected control mice were injected with 100uL of 0.9% saline i.p. at the same time. The mice were perfused at 7 months of age.

3.12 Preparation of tissue for microscopic examination

Mice were anesthetized with Rompun (5mg/kg) (Bayer Corp., Shawnee Mission, Kansas) and Ketamine (100mg/kg) (Fort Dodge Animal Health, Fort Dodge, Iowa) and perfused via the left ventricle with 10% formalin. The fixed spinal columns containing the spinal cord were isolated and demineralized in 12.5% EDTA in NaOH, pH 7.0, for one week. A total of five to seven cross sections of column containing cord were then taken from representative levels (cervical, thoracic, and lumbar), embedded in paraffin, sectioned at 4 microns, and stained with hematoxylin and eosin (H+E) for microscopic examination. Two areas of liver from each animal treated with IFN-τ 4-6 months p.i. were also prepared for examination.

3.13 Evaluation of microscopic lesions

Histological examinations of the spinal cords and livers were made without prior knowledge of experimental protocol or disease classification by two investigators independently. The liver sections were be examined for the presence of lesions. The spinal cord sections scoring was determined according to the degree of demyelination, inflammation, and status spongiosus in the meninges, white matter (ventral funiculi, lateral funiculi, dorsal funiculi), and gray matter: 0 – negative, no lesion; 0.5 – very mild – marginally identifiable lesion (0-5% area affected); 1 – mild – definitive lesion, focal and limited in scope (5-25% area affected); 2 – moderate – definitive lesion, multifocal and moderately extensive in scope (25-50% affected); 3- prominent – definitive lesion, multifocal and prominently extensive in scope (50-75%

affected); 4 – very prominent – definitive lesion, multifocal and very prominently extensive in scope (100% affected). Every slide was given an overall score for each area and region of the spinal cord. The pooled control and treated group scores as well as the mean scores were determined. The liver sections were examined and scored plus or minus for the presence or absence of lesions.

3.14 Electron microscopy

For electron microscopy analysis, mice were anesthetized with Rompun (5mg/kg) (Bayer Corp., Shawnee Mission, Kansas) and Ketamine (100mg/kg) (Fort Dodge Animal Health, Fort Dodge, Iowa) and perfused via the left ventricle with 4% paraformaldehyde/gluteraldehyde. Sodium cacodylate-trihydrate 0.2M (EM Sciences, Ft. Washington, PA) was used as a buffer after paraformaldehyde/gluteraldehyde fixation. Spinal cord sections were embedded in Epon/Araldite, sectioned at 80nm and placed on copper grids. Sections were stained with uranyl acetate and lead citrate for 12 minutes each. Sections were then viewed and photographs made using a Zeiss 10C electron microscope.

3.15 Preparation of tissue for immunostaining

Mice were anesthetized with Rompun (5mg/kg) (Bayer Corp., Shawnee Mission, Kansas) and Ketamine (100mg/kg) (Fort Dodge Animal Health, Fort Dodge, Iowa) and perfused via the left ventricle with 10% formalin. The fixed spinal columns containing the spinal cord were isolated and demineralized in a 1:1 solution, 50% formic acid: 20% sodium citrate, for 5 hours. The samples were then rinsed with tap water for 12 hours. A total of five to seven cross sections of column containing cord were

then taken from representative levels (cervical, thoracic, and lumbar), embedded in paraffin, and sectioned at 4 microns.

3.16 Immunostaining for myelin oligodendrocyte glycoprotein

Formalin fixed spinal cord sections were mounted on poly-L-lysine coated slides. Sections were deparaffinized through xylene and graded alcohols. The slides were quenched in methanol and 0.3% hydrogen peroxide for 30 min. Antigen retrieval was performed in 10mM sodium citrate pH 6.0 in a 80°C water bath for 30min. The sections were brought to room temperature and washed with double distilled H_2O (ddH_2O) and PBS. Non-specific binding was blocked using 10% normal rabbit serum (Sigma, St. Louis, MO) and the slides were then incubated for 12-14 hours at 4°C coated with myelin/oligodendrocyte Ab-1 IgM (Clone CE1) (NeoMarkers, Inc., Fremont, CA) as the primary antibody. For controls, mouse IgM (Sigma, St. Louis, MO), and PBS/2% BSA were used.

The slides were washed with PBS and PBS/2% BSA/1% triton to cut down on background and then the secondary antibody, rabbit anti-mouse horseradish peroxidase conjugated IgG (Sigma, St. Louis, MO) diluted in 5% normal rabbit serum was left to incubate for 1 hour at room temperature. After washing with PBS and Tris-HCl 0.05M pH 6.8, One 5g 3,3'Diaminobenzidine tetrahydrochloride (DAB) tablet, (ICN, Aurora, OH) diluted in 3% hydrogen peroxide and Tris-HCl 0.05M pH 6.8 was added for signal detection. The samples were then washed with ddH_2O and counter stained with Mayer's hematoxylin solution (Sigma, St. Louis, MO) for microscopic examination. See appendix for complete protocol.

3.17 Evaluation of immunostained slides

Immunostained slides of the spinal cord tissue of the following treatment groups were rated: experimental mice treated with IFN-τ 4-6 months post-infection and two control groups consisting of BeAn infected mice injected with saline and aged matched healthy mice. Sixteen to twenty slides were prepared and stained per animal in 4 micron sections with 20 microns between each slide cut. Scoring was determined according to the degree of demyelination detected via immunostaining for MOG, in the meninges, white matter (ventral funiculi, lateral funiculi, dorsal funiculi), and gray matter: 0 – negative, no lesion; 0.5 – very mild – marginally identifiable lesion (0-5% area affected); 1 – mild – definitive lesion, focal and limited in scope (5-25% area affected); 2 – moderate – definitive lesion, multifocal and moderately extensive in scope (25-50% affected); 3- prominent – definitive lesion, multifocal and prominently extensive in scope (50-75% affected); 4 – very prominent – definitive lesion, multifocal and very prominently extensive in scope (100% affected). Every slide was given an overall score for each region of the spinal cord. The pooled control and treated group scores as well as the mean scores were determined.

3.18 Statistical analysis

Standard deviations as calculated via ANCOVA from the mean and standard error were used to calculate error bars and determine statistical significance in all the histological analyses.

Results

3.19 Correlation between histology and clinical score rating

Mean histological and clinical scores for mice from all treatment groups (n=120 mice) were plotted and the R^2 value was determined using Microsoft Excel. A R^2 value of 0.9039 established the connection between clinical scores and histological lesion presentation (figure 3.1). This validates the subjective clinical scoring system.

3.20 Evaluation of microscopic lesions in H+E stained slides

The overall affect of interferon-tau treatment at 4-6 months p.i. was evaluated by rating lesion presentation throughout cervical, thoracic, and lumbar regions of the spinal cords of treated and non-treated infected mice. The location of the lesions was determined and classified as either meningeal, gray matter, or white matter to include the dorsal, ventral, and lateral funiculi. Using histologic score values, figure 3.2 depicts that slight inflammation and status spongiosus that was observed in the gray matter of the treated mice and not in the infected controls, $p<0.05$. As seen in figure 3.3, meningeal inflammation decreased in interferon-tau treated mice when compared to infected controls, $p<0.05$. Demyelination and inflammation was significantly decreased in the dorsal funiculi of interferon-tau treated mice, $p<0.05$ (figure 3.4). Similar affects were also seen in the ventral funiculi accompanied with a decrease in status spongiosus, $p<0.05$ (figure 3.5). No significant differences were seen with interferon-tau treatment in the lateral funiculi, $p>0.01$ (figure 3.6). A decrease in inflammation and demyelination was observed throughout the cervical, thoracic, and

lumbar regions of the spinal cords of the 4-6 month treated mice, $p<0.05$ (figures 3.7 and 3.8).

Figures 3.9 and 3.10 display the amount of extensive demyelination and status spongiosus observed in mice treated from 3 days pre-infection to 60 days post-infection with interferon-tau. Status spongiosus and inflammation in the meninges and white matter are evident in the mice treated from 2-6 months with interferon-tau (figures 3.11 and 3.12).

Figures 3.13-3.17 are from mice treated from 4-6 months with interferon-tau. Some inflammation and demyelination are present, though lesion presentation is minimal when compared to virus-infected controls (figures 3.20 and 3.21) where perivascular cuffing is seen in addition to meningeal inflammation, status spongiosus and demyelination in the white matter. Mice treated with methylprednisolone, a known anti-inflammatory, 5-6 months post-infection had a decrease in inflammation though status spongiosus and demyelination are still present (figures 3.18 and 3.19). Non-infected, non-treated controls are shown in figures 3.22 and 3.23. No liver lesions were detected.

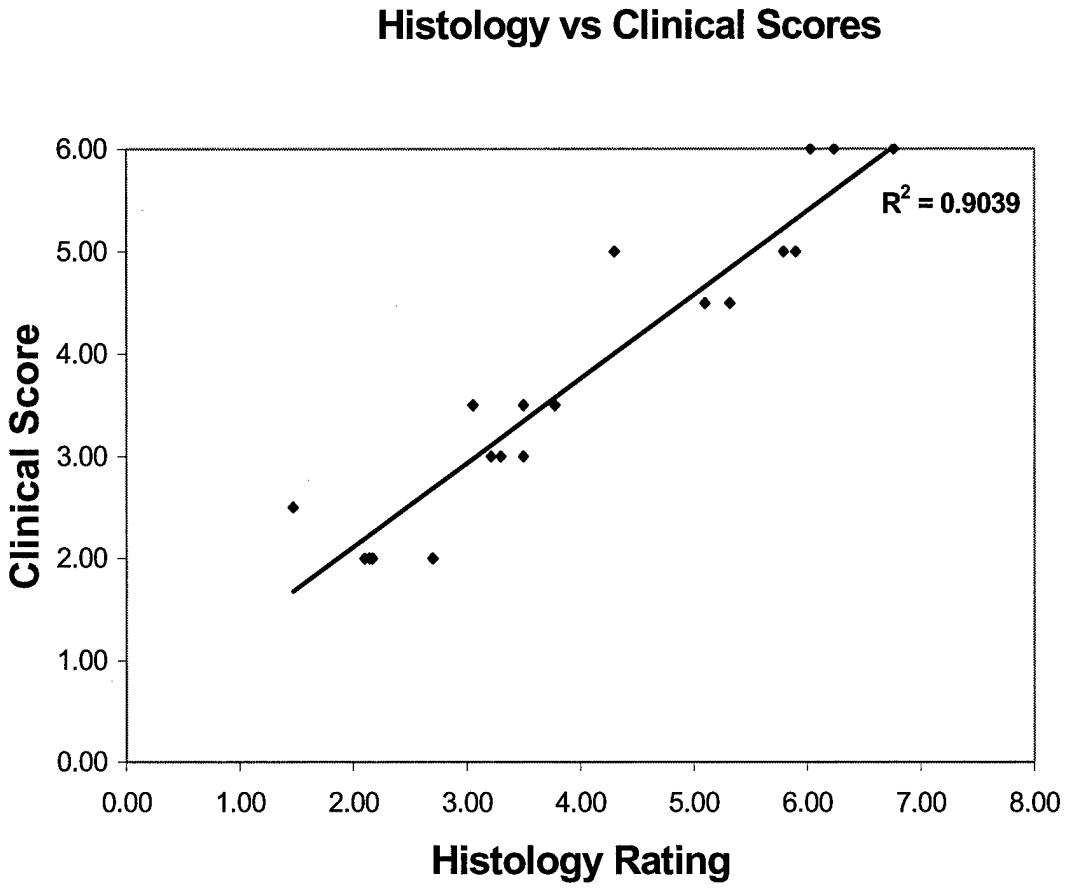

Figure 3.1: Correlation graph of histological v. clinical scores.
Correlation graph between the mean histological rating of H+E stained spinal cord slides per animal and clinical scores of animals infected with BeAn and treated with IFN-τ or saline.

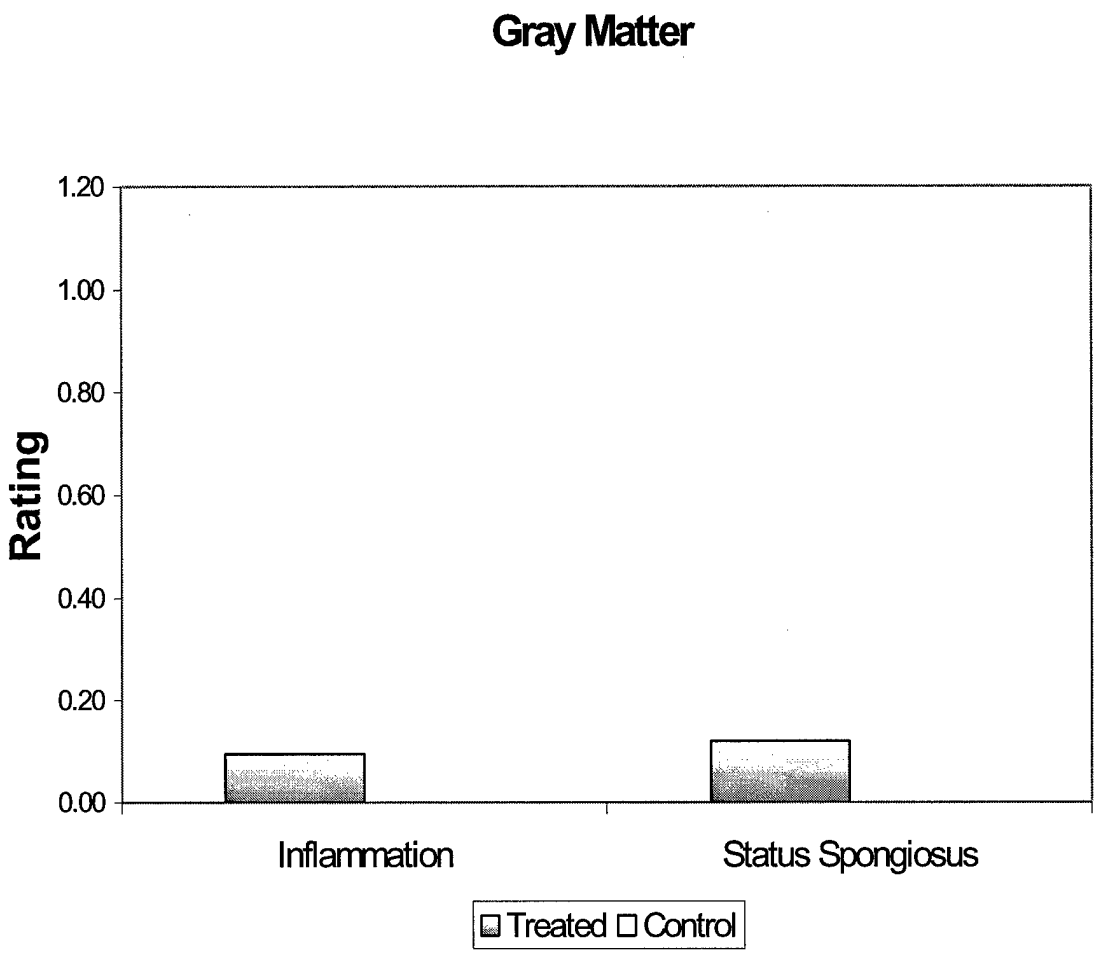

Figure 3.2: Histology rating of gray matter lesions. Mean histological scores of H+E stained spinal cord sections illustrating gray matter lesions of mice infected with BeAn and treated with IFN-τ 4-6 months p.i. or saline virus-infected controls.

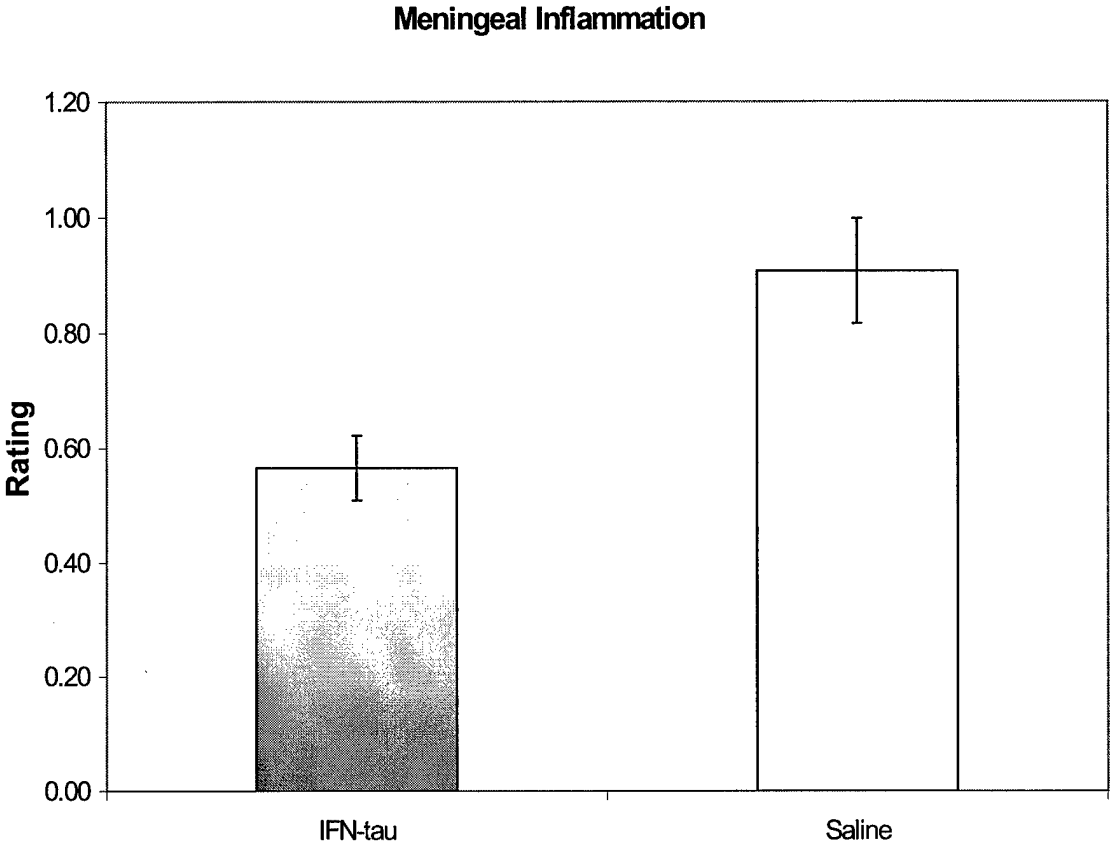

Figure 3.3: Histology rating of meningeal inflammation. Mean histological scores of H+E stained spinal cord sections illustrating meningeal lesions of mice infected with BeAn and treated with IFN-τ 4-6 months p.i. or saline virus-infected controls.

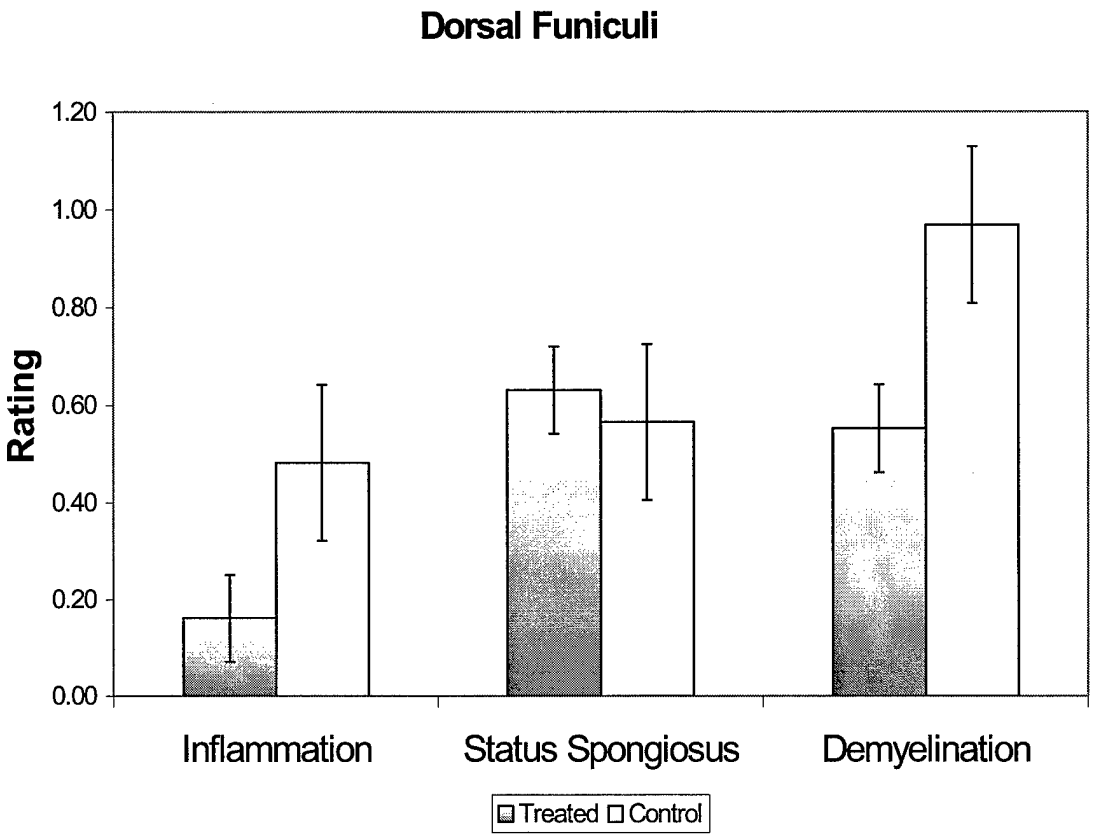

Figure 3.4: Histology rating of dorsal funiculi lesions. Mean histological scores of H+E stained spinal cord sections illustrating dorsal funiculi lesions of mice infected with BeAn and treated with IFN-τ 4-6 months p.i. or saline virus-infected controls.

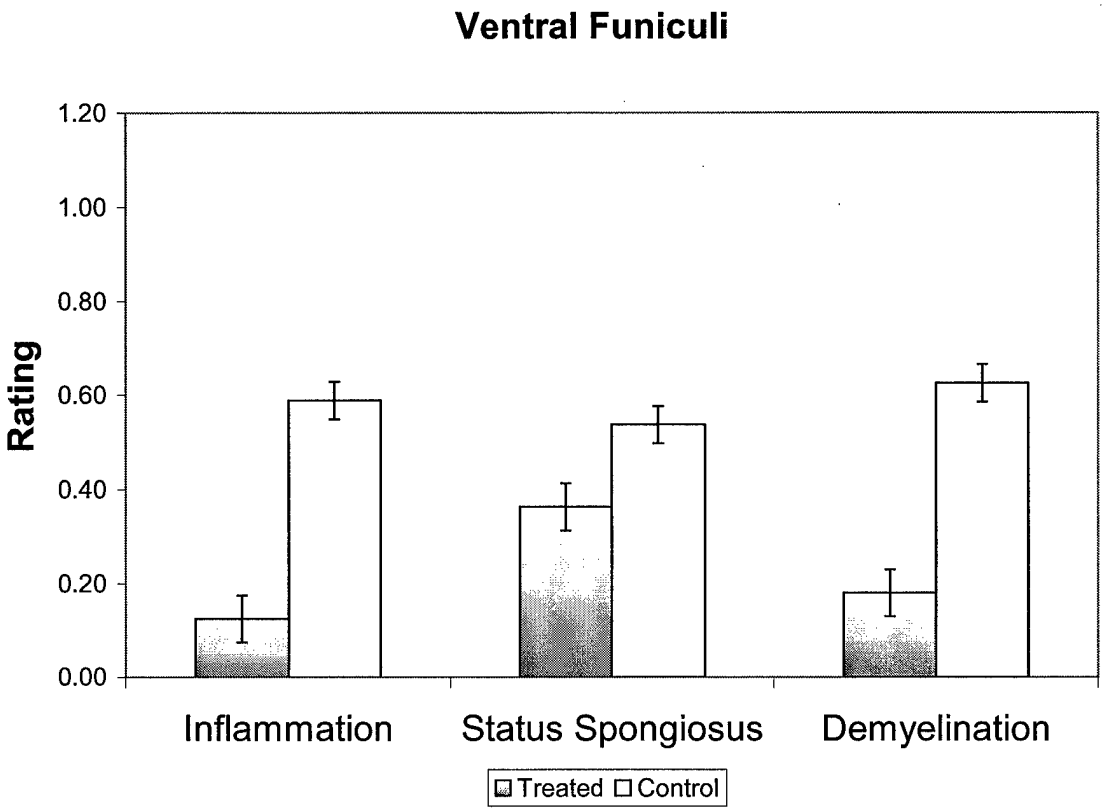

Figure 3.5: Histology rating of ventral funiculi lesions. Mean histological scores of H+E stained spinal cord sections illustrating ventral funiculi lesions of mice infected with BeAn and treated with IFN-τ 4-6 months p.i. or saline virus-infected controls.

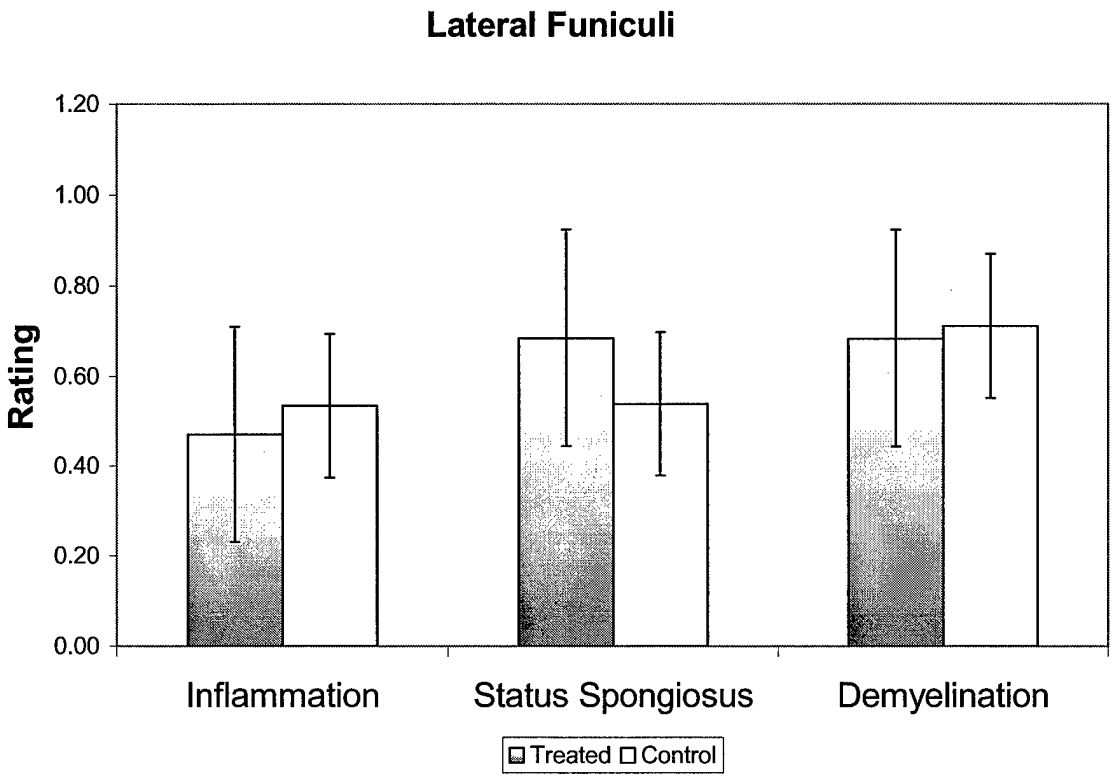

Figure 3.6: Histology rating of lateral funiculi lesions. Mean histological scores of H+E stained spinal cord sections illustrating lateral funiculi lesions of mice infected with BeAn and treated with IFN-τ 4-6 months p.i. or saline virus-infected controls.

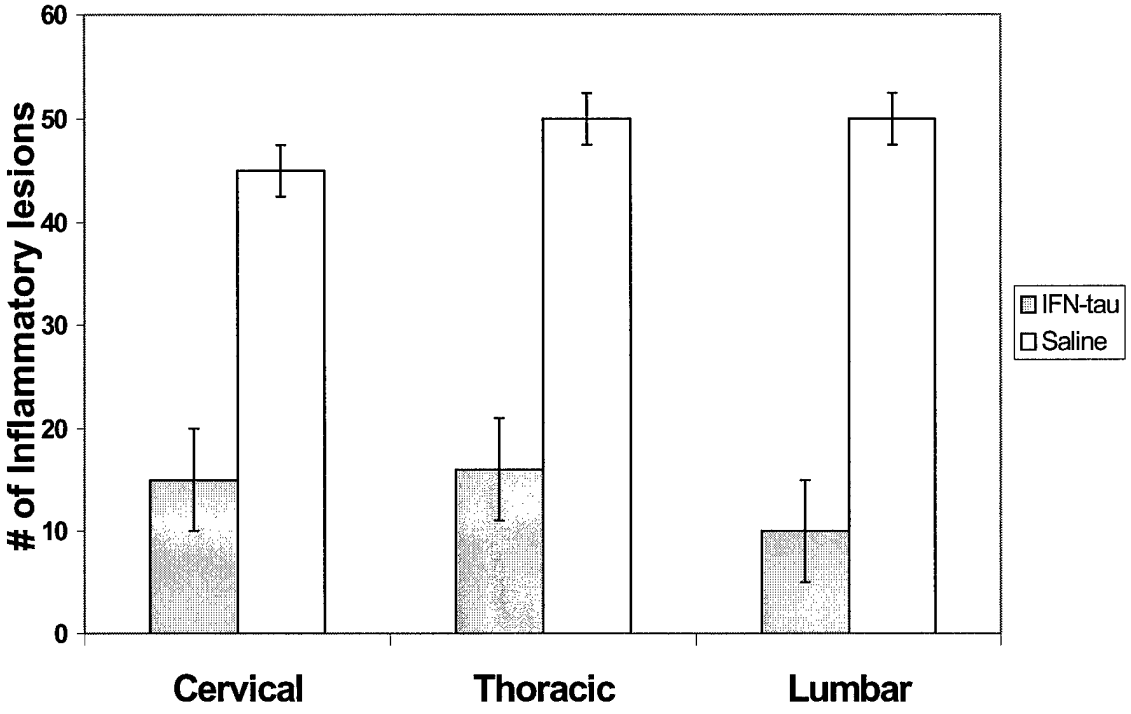

Figure 3.7: H+E inflammation scores through the cervical, thoracic, and lumbar regions of the spinal cord. Mean histological rating of inflammation in spinal cord sections of mice treated with IFN-τ 4-6 months p.i. or saline virus-infected controls. Fifty tissue sections were rated per treatment group per level of the spinal cord.

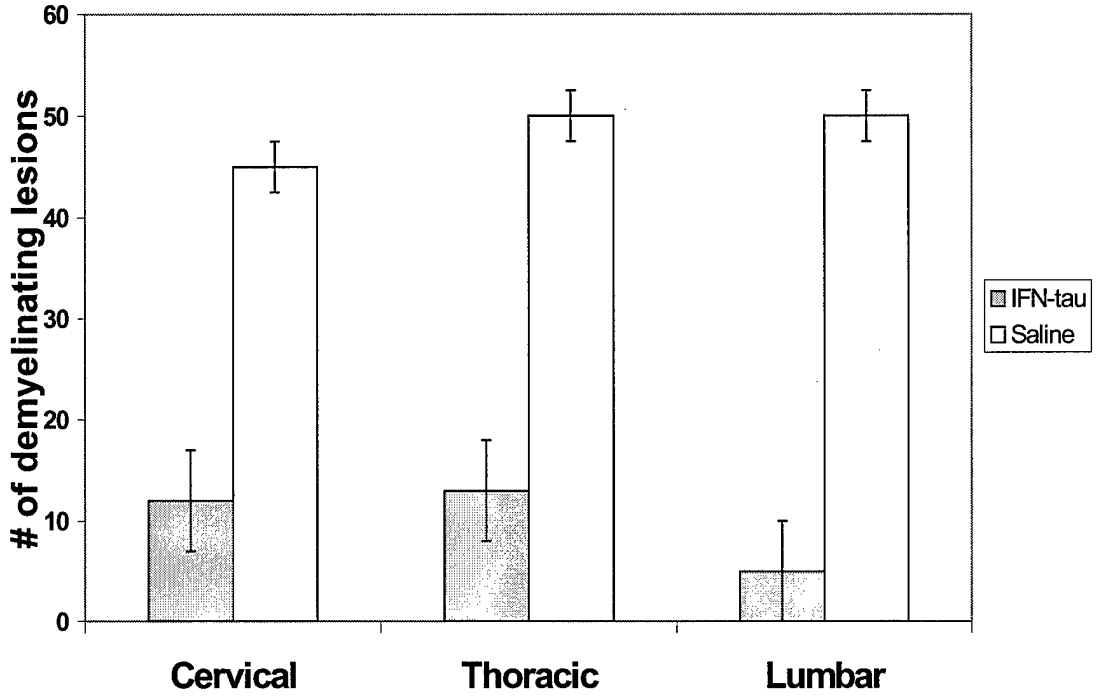

Figure 3.8: Demyelination through the cervical, thoracic, and lumbar regions of the spinal cord. Mean histological rating of demyelination in spinal cord sections of mice treated with IFN-τ 4-6 months p.i. or virus-infected saline controls. Slides stained for CNS myelin were used and 50 tissue sections were rated per treatment group per level of the spinal cord.

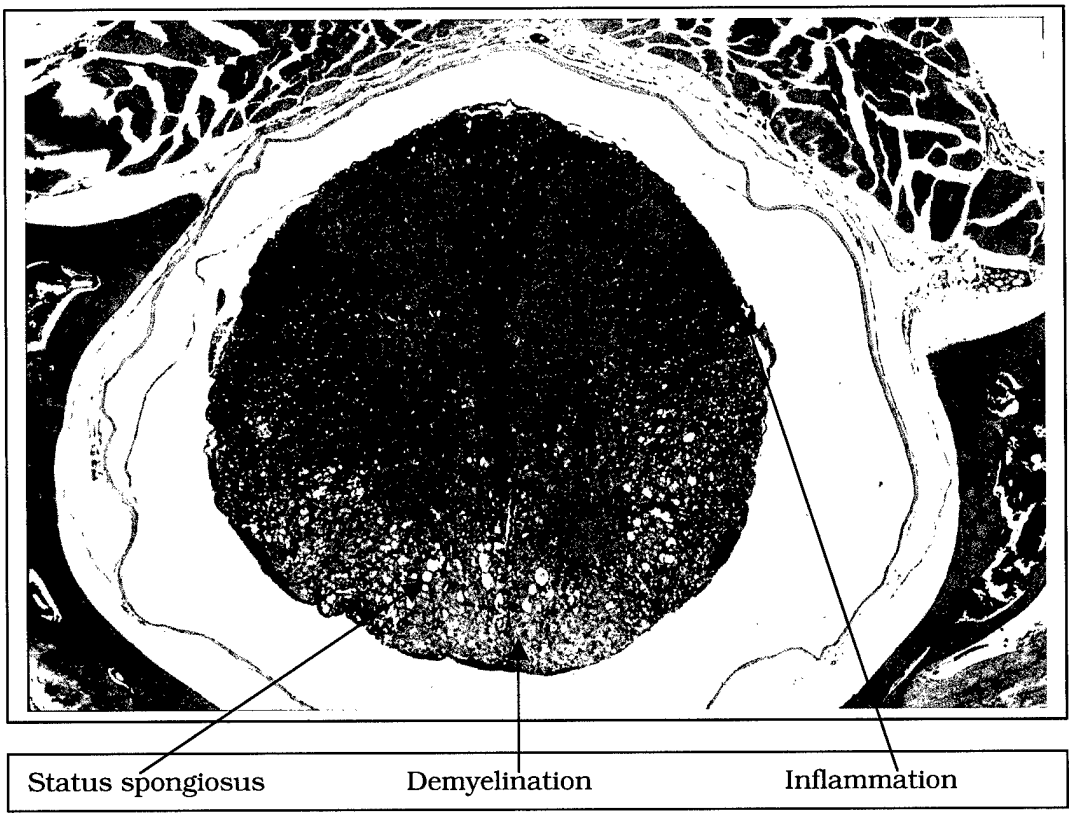

Figure 3.9: H+E stained slide of a mouse pretreated from 3 days pre-infection to 60 days post-infection with interferon-tau. 40X magnification. Note status spongiosus and demyelination present in the ventral and lateral funiculi as well as inflammation in the meninges.

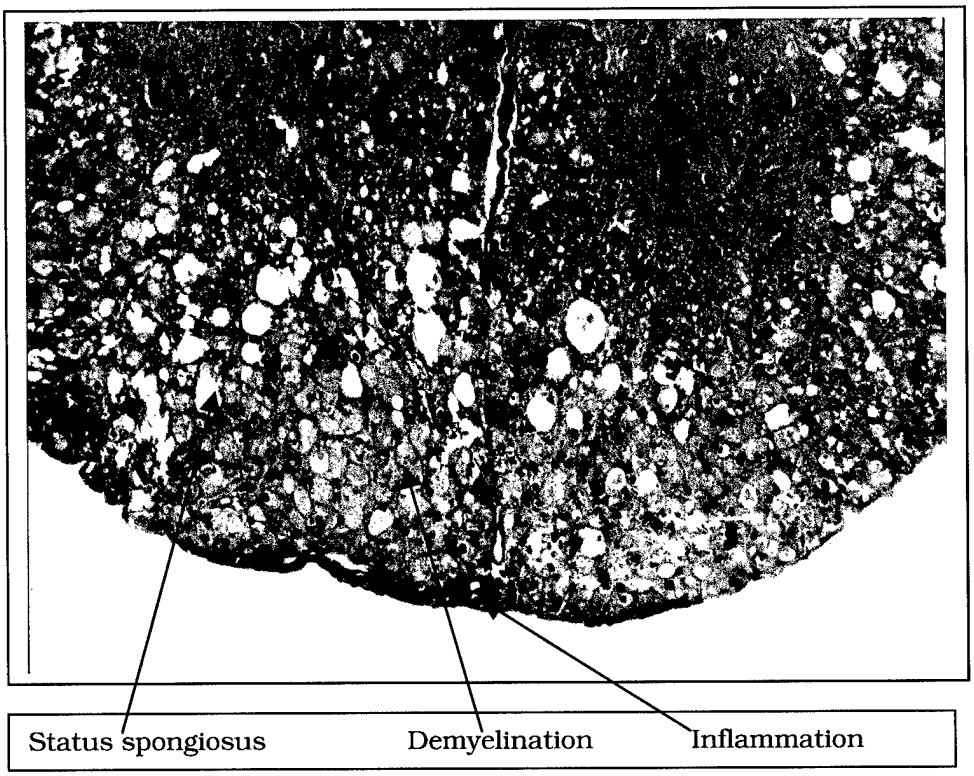

Figure 3.10: H+E stained slide of a mouse pretreated from 3 days pre-infection to 60 days post-infection with interferon-tau. 240X magnification. Note status spongiosus and demyelination present in the ventral and lateral funiculi as well as inflammation in the meninges.

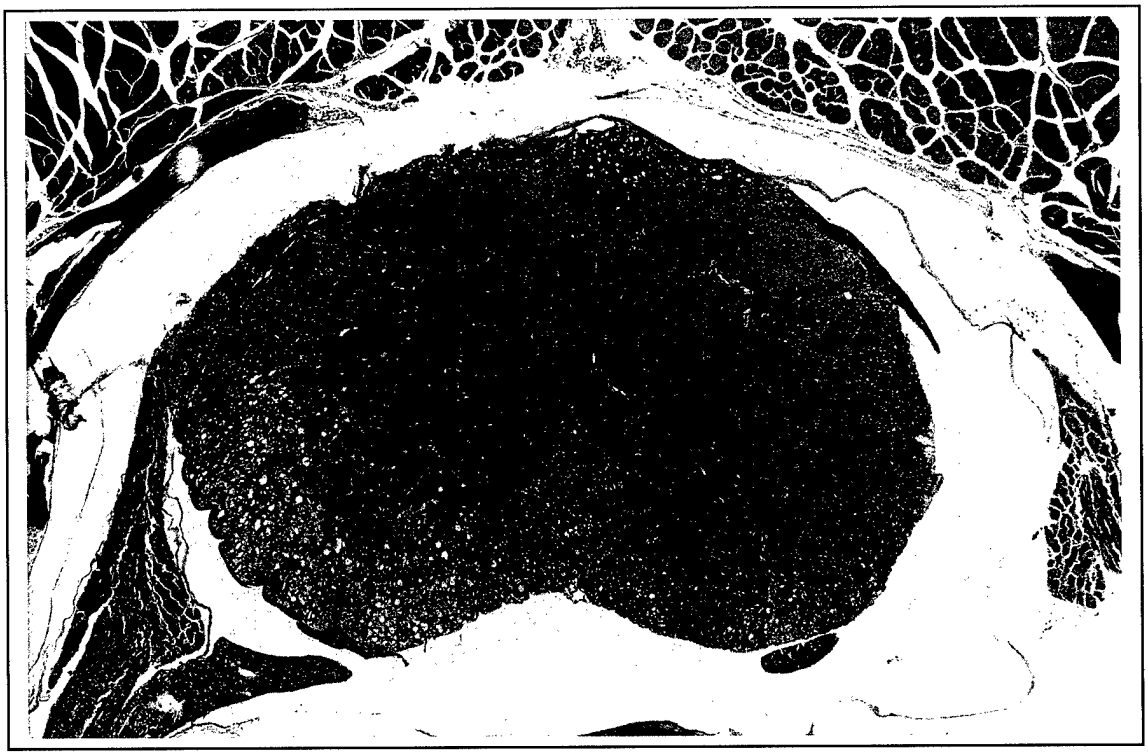

Figure 3.11: H+E stained slide of a mouse treated from 2 to 6 months post-infection with interferon-tau. 40X magnification. Note status spongiosus and demyelination present in the ventral and lateral funiculi as well as inflammation in the meninges.

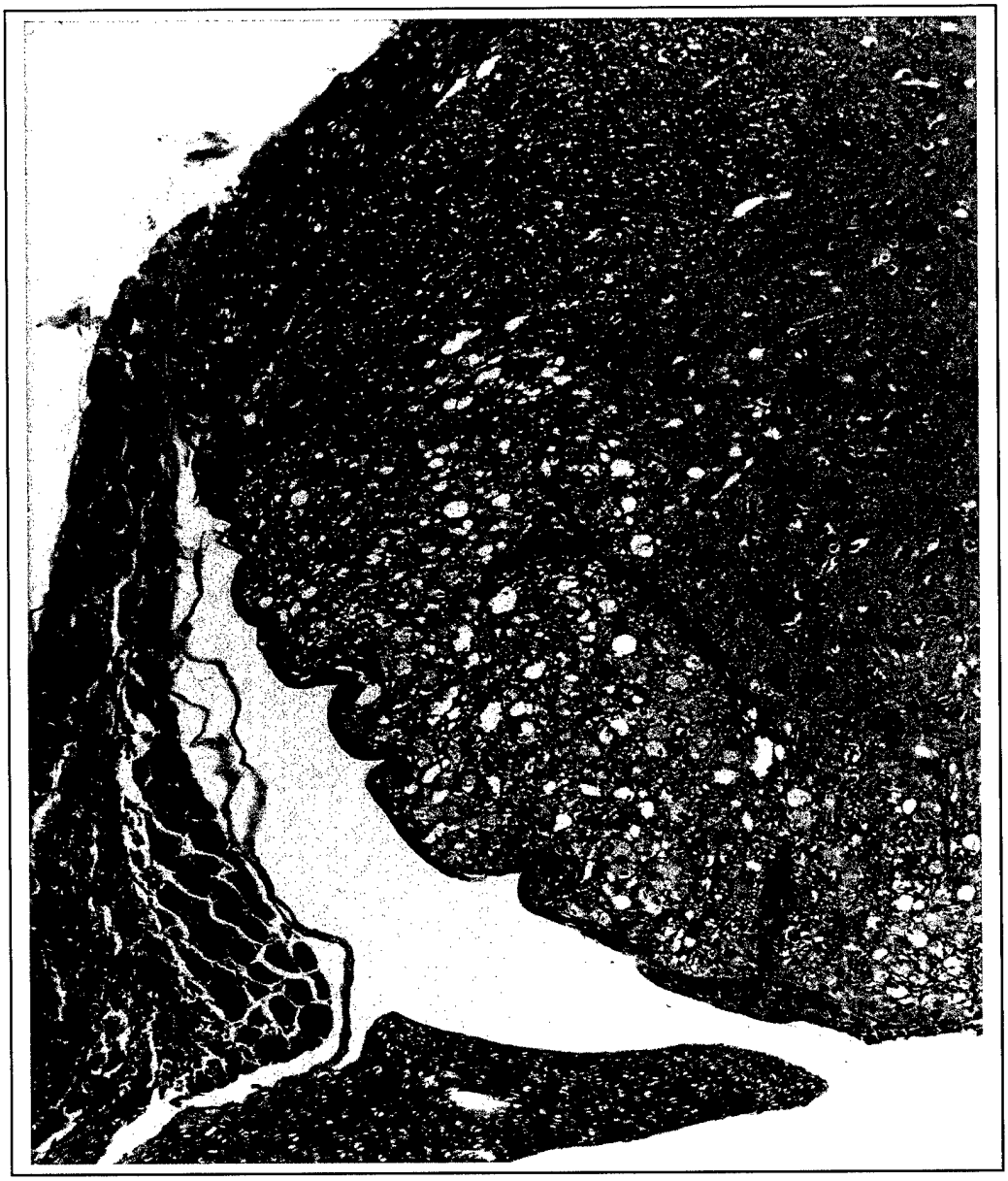

Figure 3.12: H+E stained slide of a mouse treated from 2 to 6 months post-infection with interferon-tau. 240X magnification. Note status spongiosus and demyelination present in the ventral and lateral funiculi as well as inflammation in the meninges.

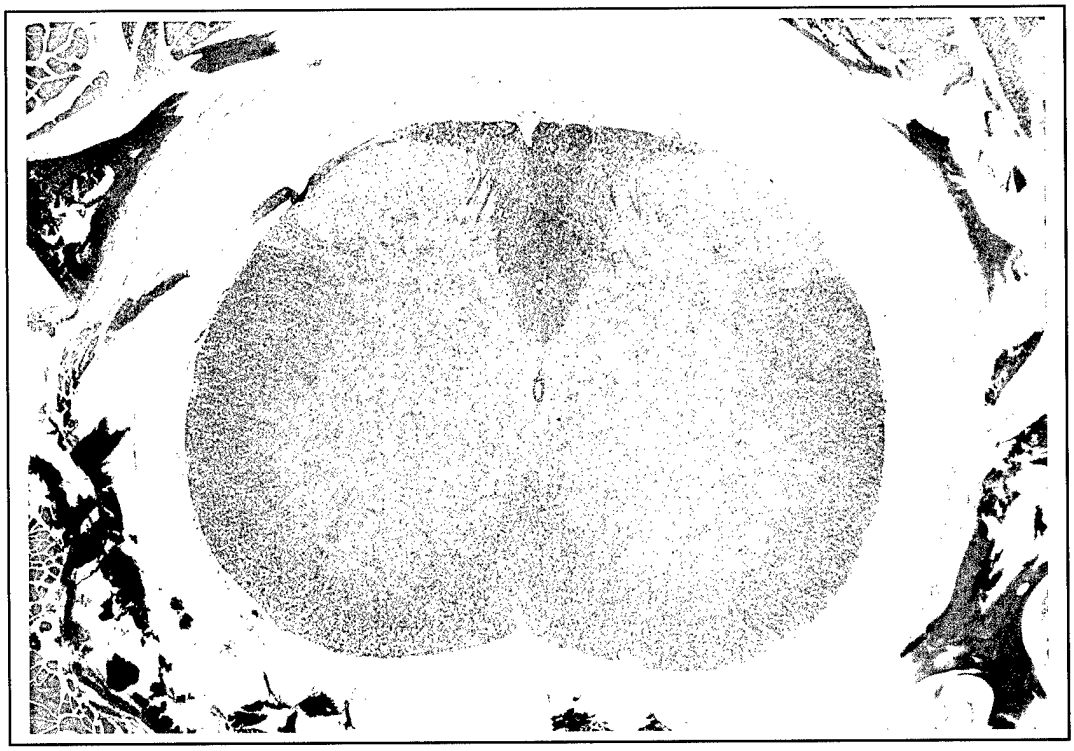

Figure 3.13: H+E stained slide of a mouse treated from 4 to 6 months post-infection with interferon-tau. 40X magnification. Note lack of status spongiosus and demyelination present in the ventral and lateral funiculi as well as no inflammation in the meninges.

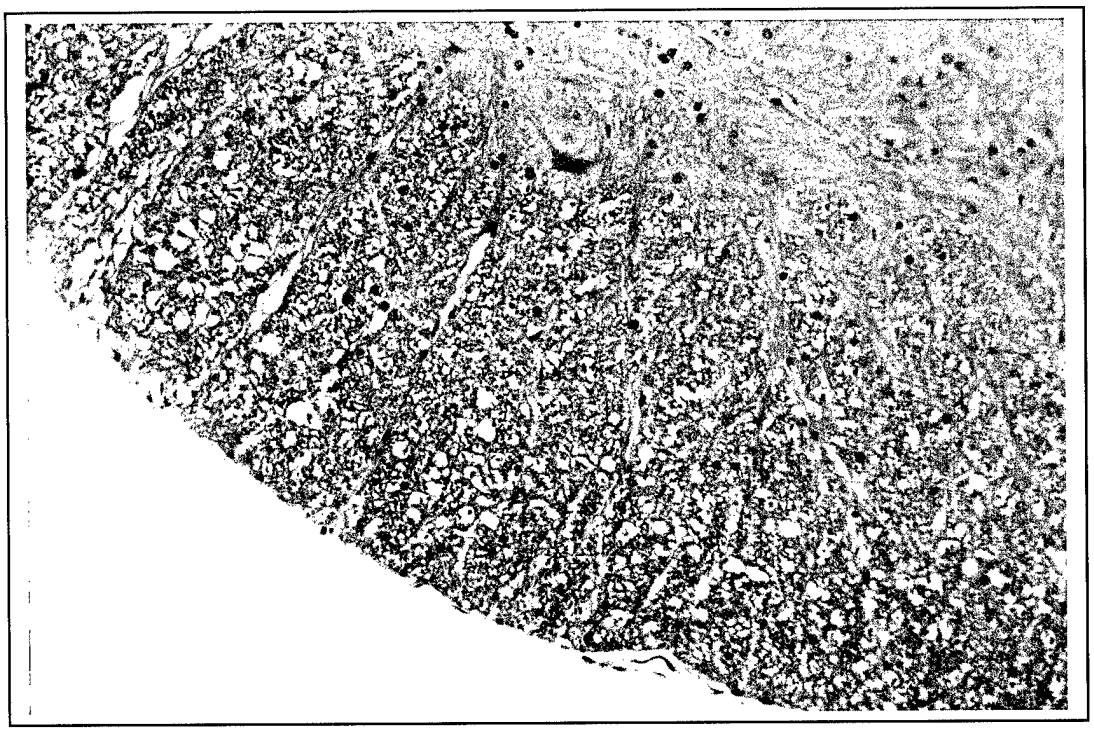

Figure 3.14: H+E stained slide of a mouse treated with interferon-tau from 4 to 6 months post-infection. 240X magnification. Note lack of status spongiosus and demyelination present in the ventral funiculi as well as no inflammation in the meninges.

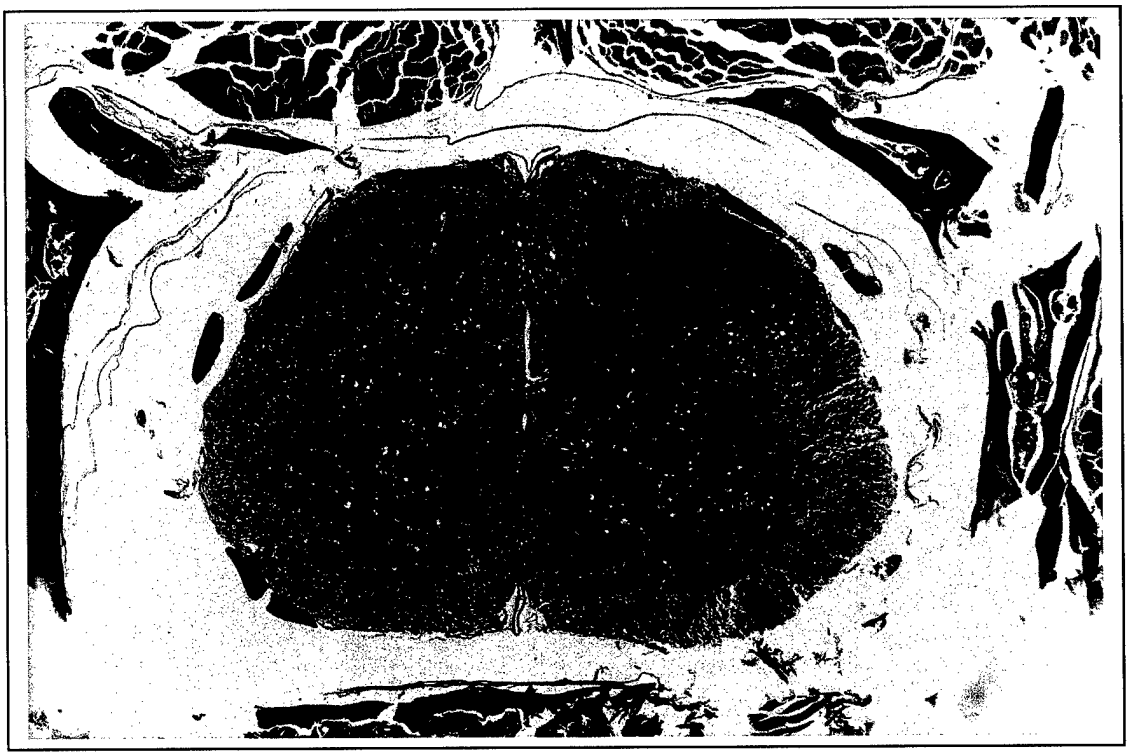

Figure 3.15: H+E stained slide of the mouse with the worst clinical score treated from 4 to 6 months post-infection with interferon-tau. 40X magnification. Note slight status spongiosus and demyelination present in the ventral and lateral funiculi as well as inflammation in the meninges.

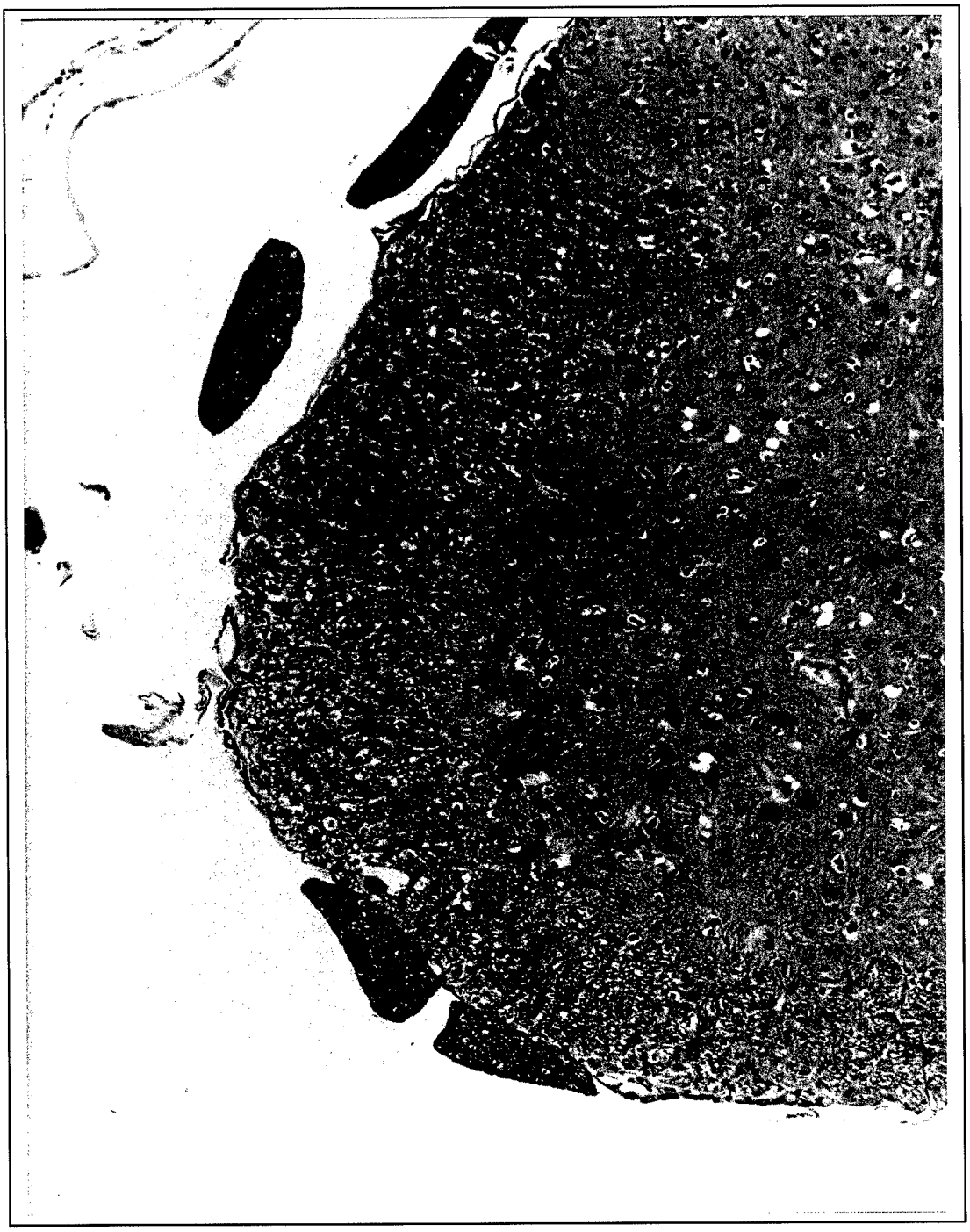

Figure 3.16: H+E stained slide of the mouse with the worst clinical score treated from 4 to 6 months post-infection with interferon-tau. 200X magnification. Note slight status spongiosus and demyelination present in the ventral funiculi as well as inflammation in the meninges.

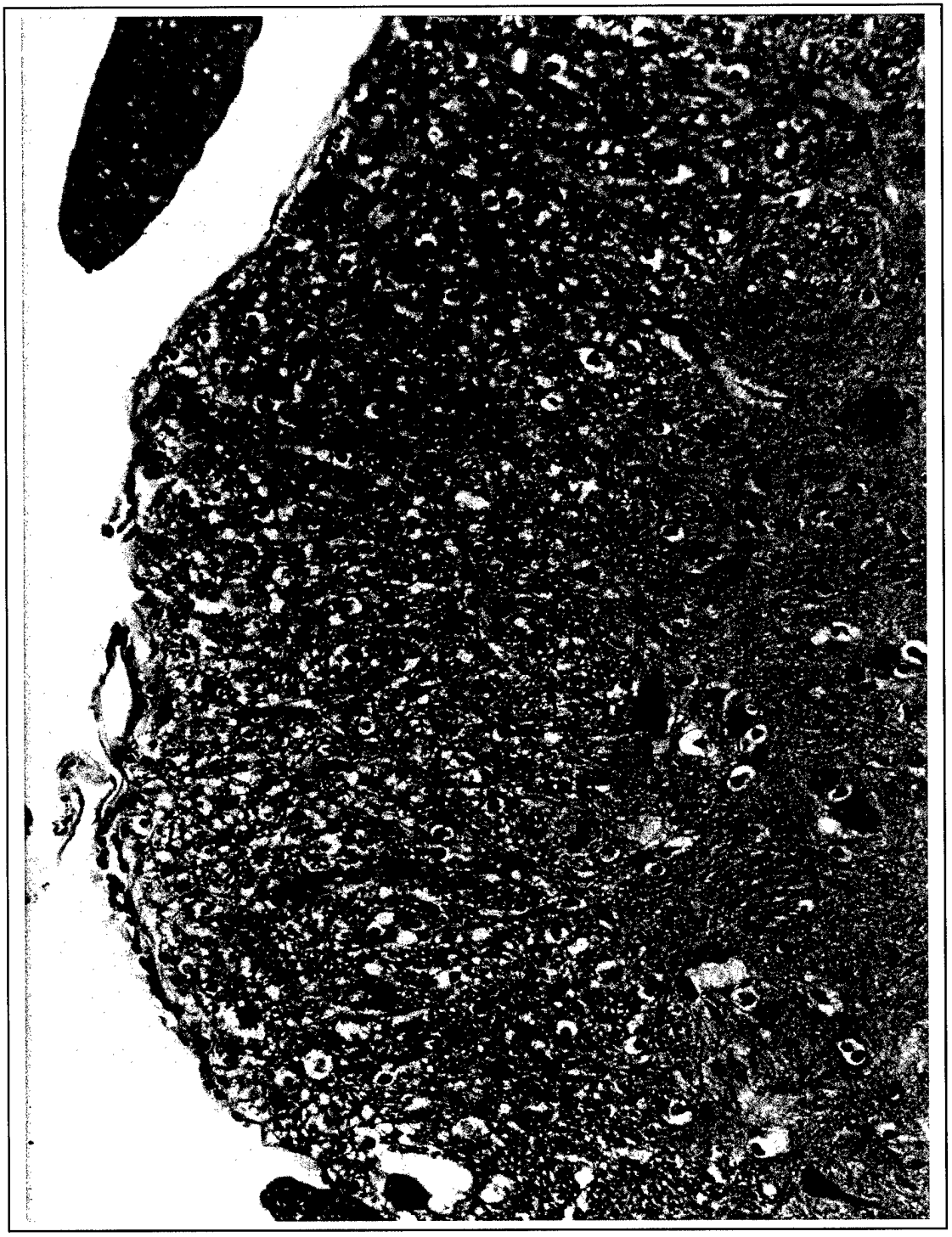

Figure 3.17: H+E stained slide of the mouse with the worst clinical score treated from 4 to 6 months post-infection with interferon-tau. 240X magnification. Note slight status spongiosus and demyelination present in the ventral funiculi as well as inflammation in the meninges.

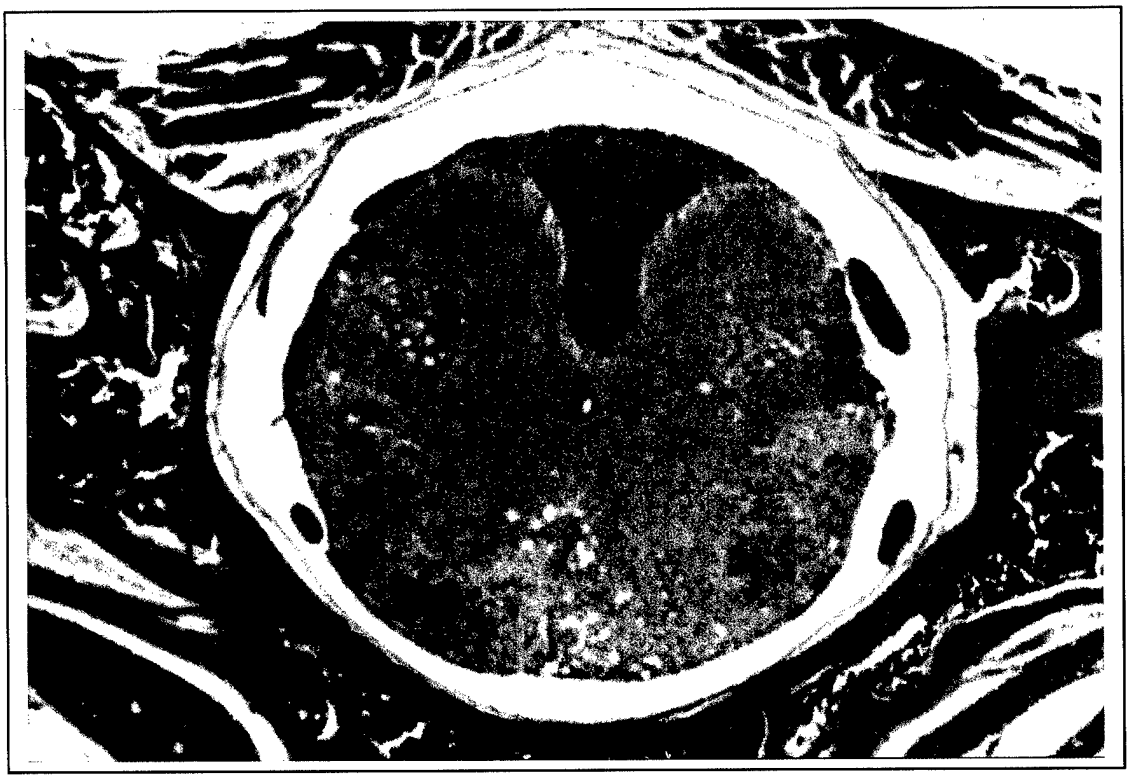

Figure 3.18: H+E stained slide of a mouse treated with methylprednisolone from 5 to 6 months post-infection. 40X magnification. Note extensive status spongiosus and demyelination present in the ventral and lateral funiculi as well as inflammation in the white matter and meninges.

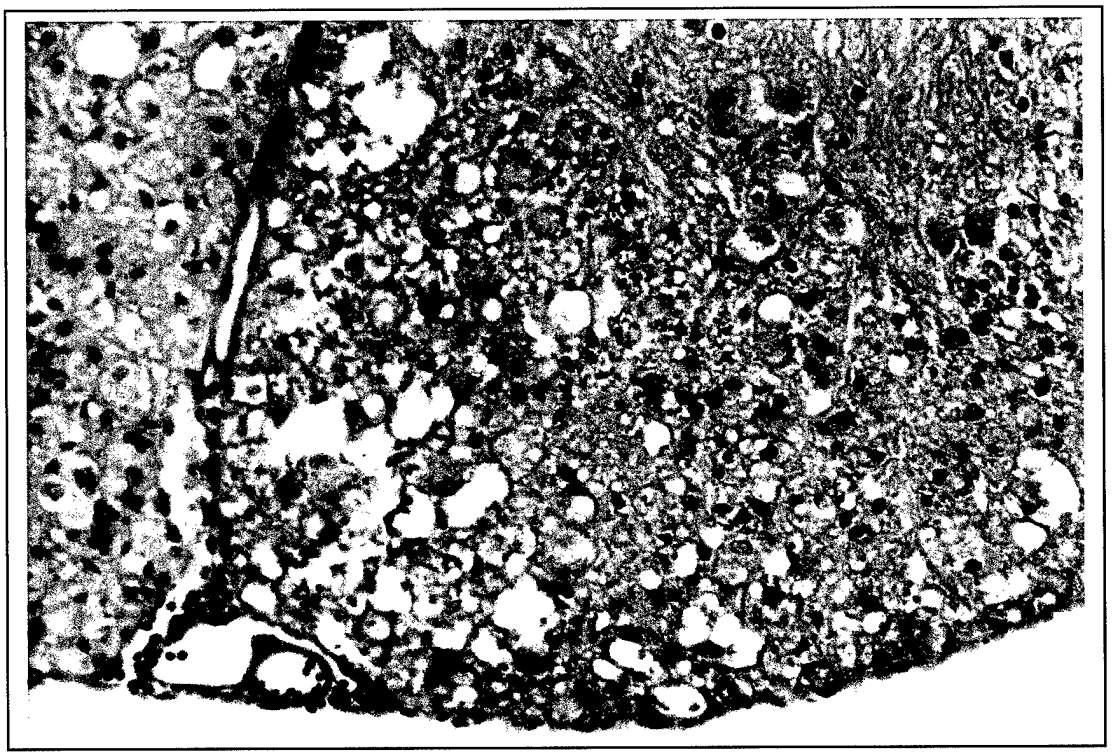

Figure 3.19: H+E stained slide of a mouse treated with methylprednisolone from 5 to 6 months post-infection. 240X magnification. Note extensive status spongiosus and demyelination present in the ventral funiculi as well as inflammation in the white matter and meninges.

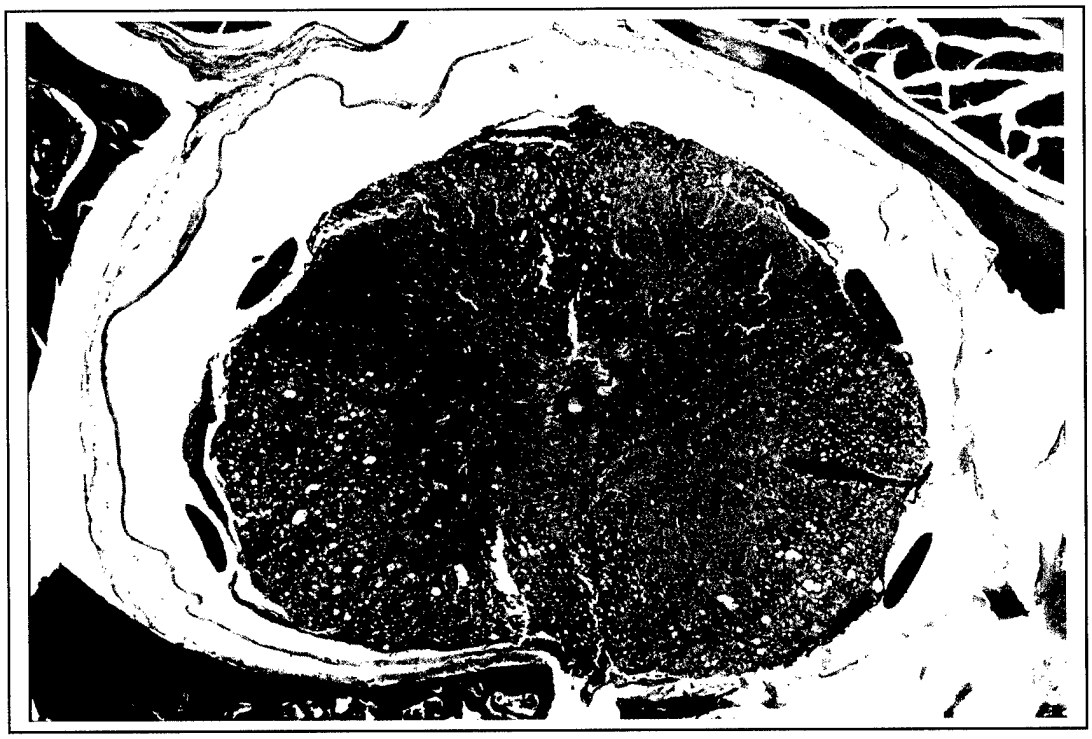

Figure 3.20: H+E stained slide of a mouse infected with virus and given saline ip from 4-6 months post-infection. 40X magnification. Note status spongiosus and demyelination present in the ventral and lateral funiculi as well as inflammation in the meninges and perivascular cuffing.

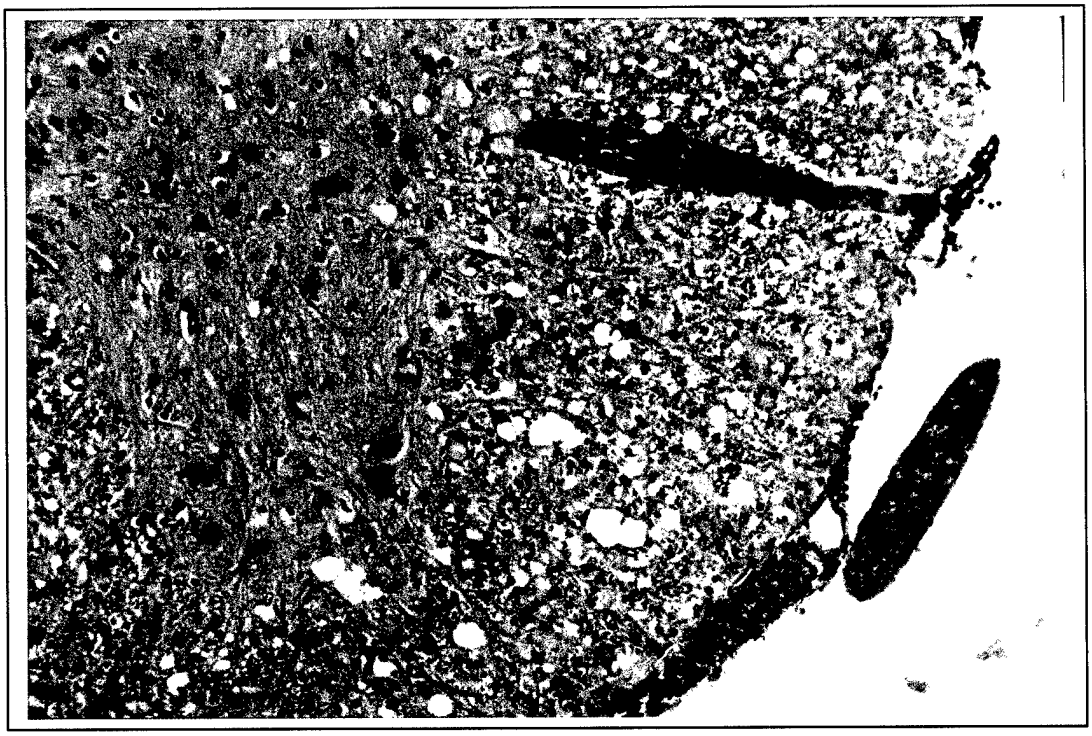

Figure 3.21: H+E stained slide of a mouse infected with virus and given saline ip from 4-6 months post-infection. 240X magnification. Note status spongiosus and demyelination present in the lateral funiculus as well as inflammation in the meninges and perivascular cuffing.

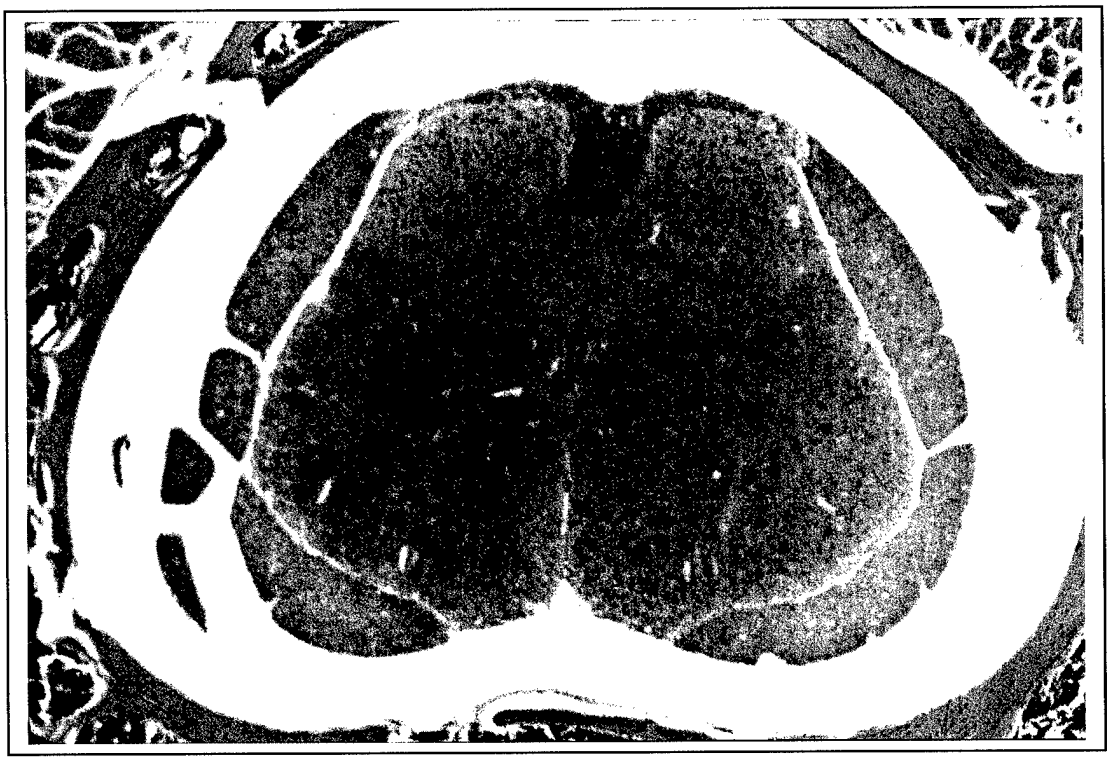

Figure 3.22: H+E stained slide of a non-infected mouse. 40X magnification. Note uninterupted staining through out the section.

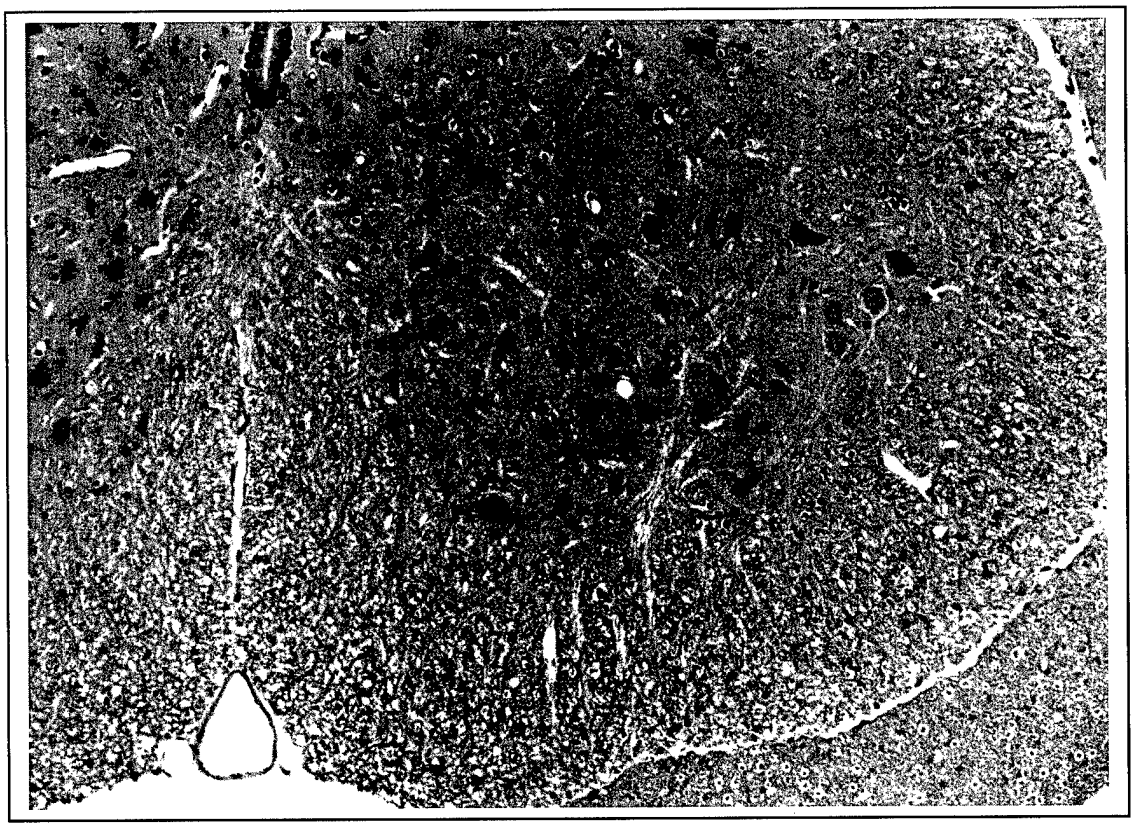

Figure 3.23: H+E stained slide of a non-infected mouse. 100X magnification. Note uninterupted staining through out the section.

3.21 Evaluation of immunostained slides

Infected mice with or without interferon-tau treatment from 4-6 months post-infection and non-infected, control mice were sacrificed. The spinal cords were sectioned as mentioned previously. All three groups had paraffin embedded spinal cord sections immunostained for CNS myelin throughout the cords. Lumbar sections are shown in the following figures. Figures 3.24 and 3.25 show the myelin-specific staining of a mouse treated from 4-6 months p.i. with interferon-tau. Figures 3.26 and 3.27 are the H+E stained serial sections of the same mouse. Notice the uniformity of both staining procedures throughout the sections. Figure 3.28 is the immunostaining of a spinal cord section from the mouse with the most severe clinical score from the 4-6 month interferon-tau treated group. In this section, the majority of the white matter retains myelin stain. An H+E stained section from the same mouse follows in figure 3.29. An immunostained section from a non-infected control mouse follows in figure 3.30. Notice the marked reduction in myelin staining in figure 3.31 taken from a viral infected mouse that was not treated with interferon-tau. The non-specific staining of the menegies in figure 3.31 is interrupted as insignificant. The H+E section of the same non-treated, virus-infected mouse also displays extensive demyelination (figure 3.32).

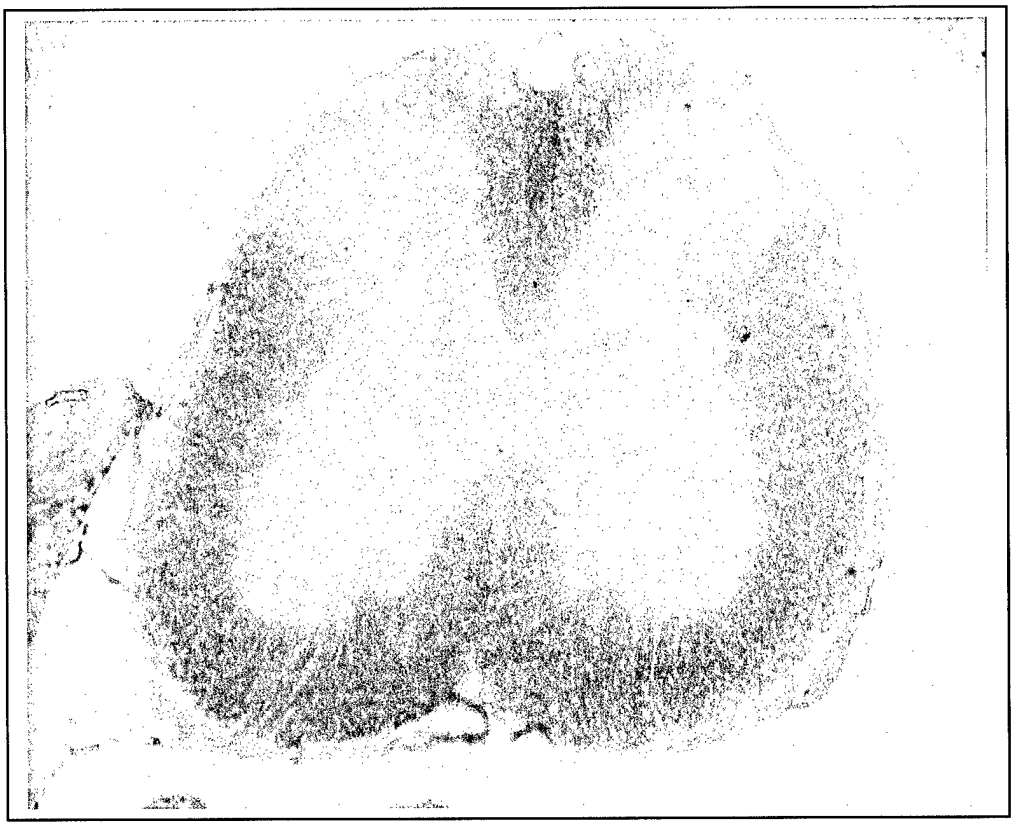

Figure 3.24: Immunostaining for CNS myelin of an infected mouse treated with interferon-tau from 4-6 months post-infection. Magnification 40X. Notice the darker staining of the white matter.

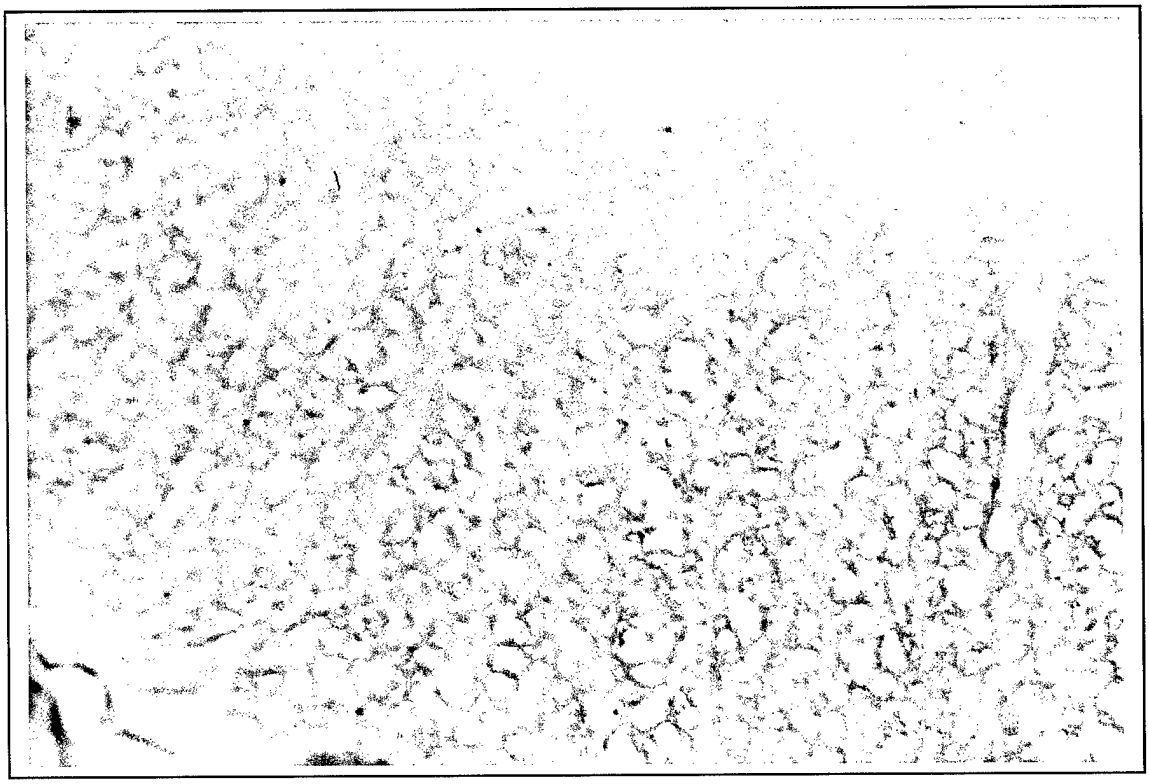

Figure 3.25: Immunostaining for CNS myelin of an infected mouse treated with interferon-tau from 4-6 months post-infection. Magnification 240X. Notice the darker staining of the myelin in the white matter.

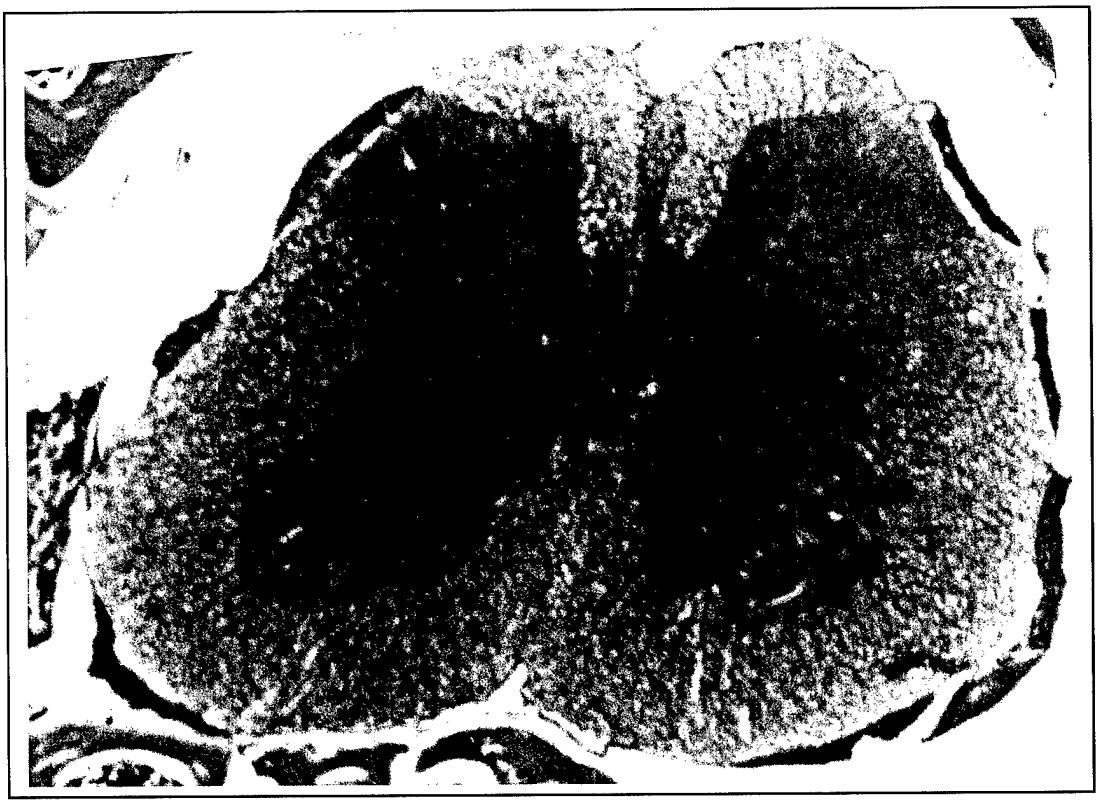

Figure 3.26: H+E staining of a serial section of an infected mouse treated with interferon-tau from 4-6 months post-infection. Magnification 40X. Notice the lighter staining of the white matter where the myelin is located.

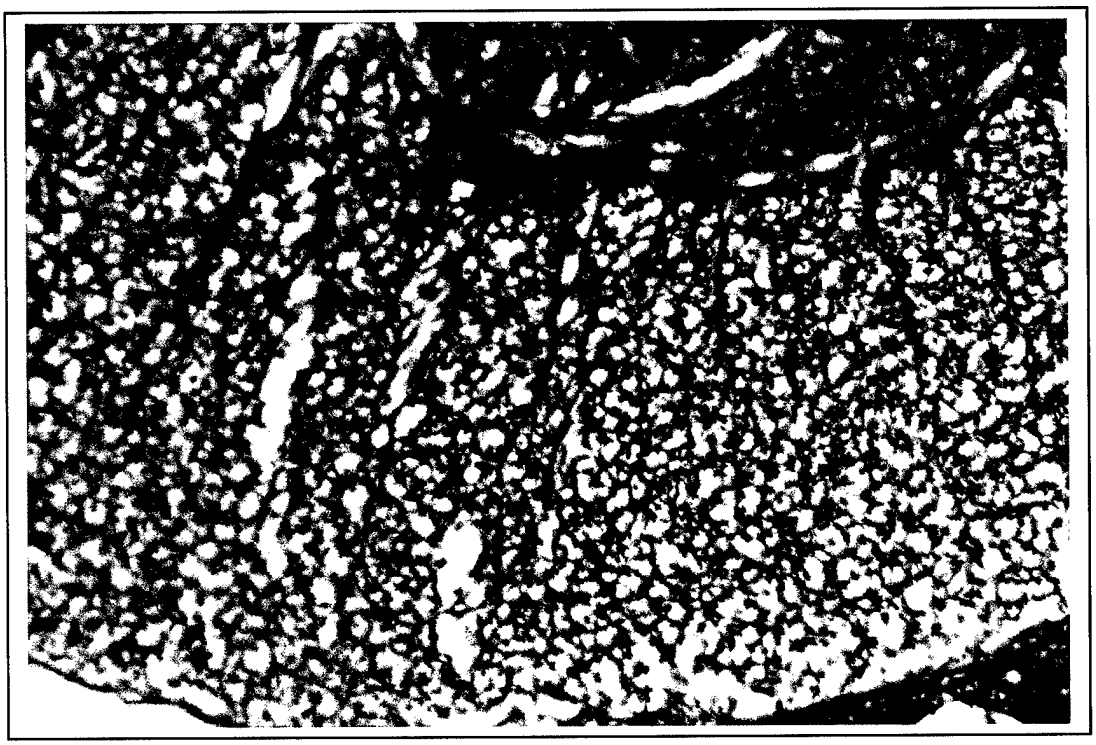

Figure 3.27: H+E staining of a serial section of an infected mouse treated with interferon-tau from 4-6 months post-infection. Magnification 240X. Notice the lighter staining of the white matter where the myelin is located.

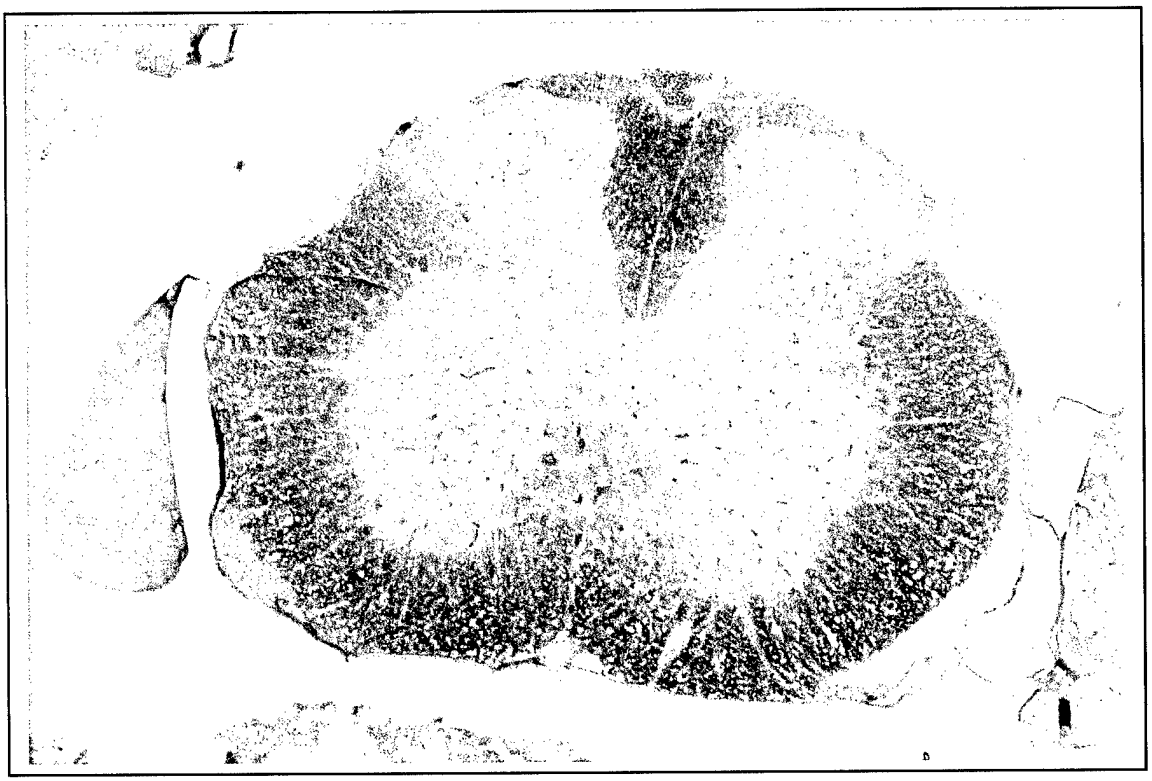

Figure 3.28: Immunostaining for CNS myelin of an infected mouse with the worst clinical score treated with interferon-tau from 4-6 months post-infection. Magnification 40X. Notice the darker staining of the white matter.

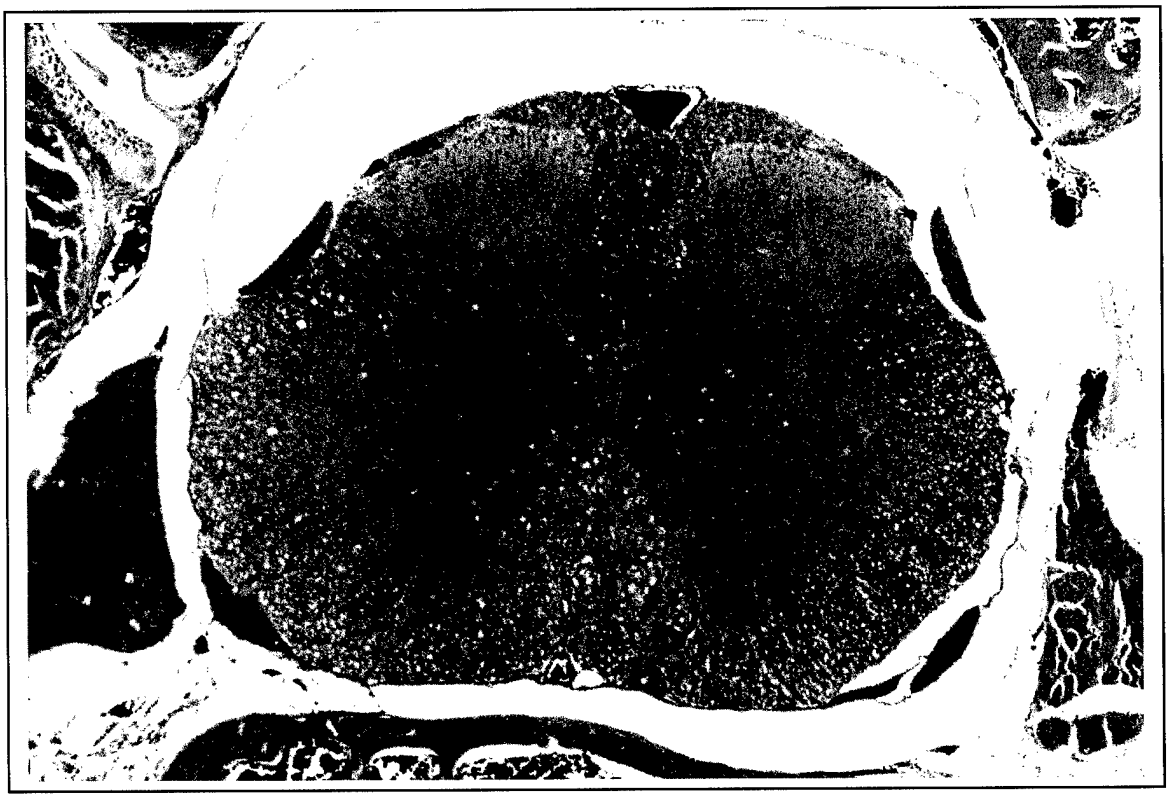

Figure 3.29: H+E staining of a serial section of an infected mouse with the worst clinical score treated with interferon-tau from 4 to 6 months post-infection. Magnification 40X. Notice the lighter staining of the white matter.

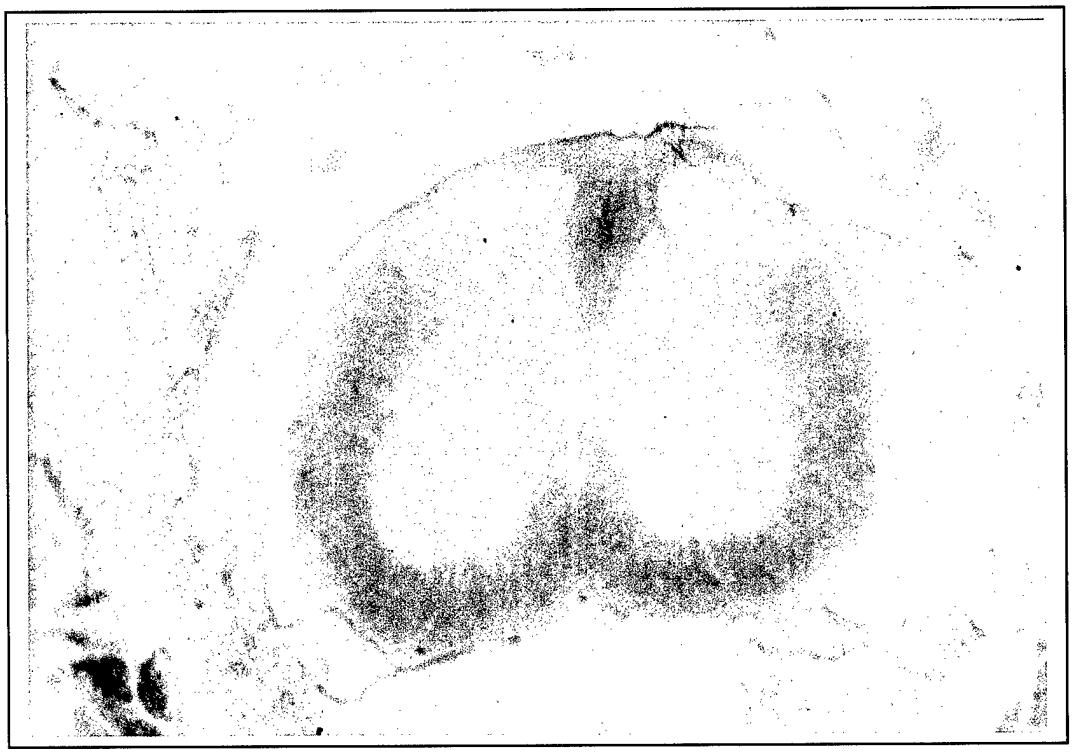

Figure 3.30: Immunostaining for CNS myelin of a non-infected mouse. Magnification 40X. Notice the darker staining of the white matter.

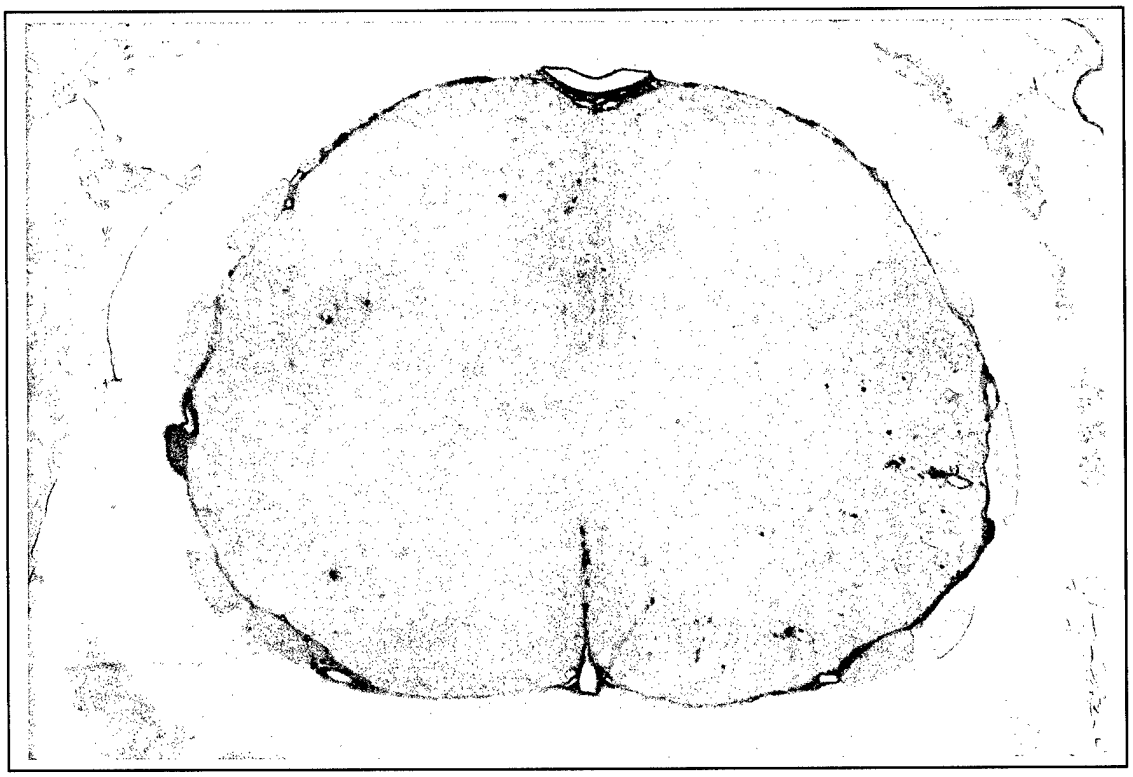

Figure 3.31: Immunostaining for CNS myelin of an infected mouse treated with saline from 4-6 months post-infection. Magnification 40X. Notice the lack of darker staining of the white matter.

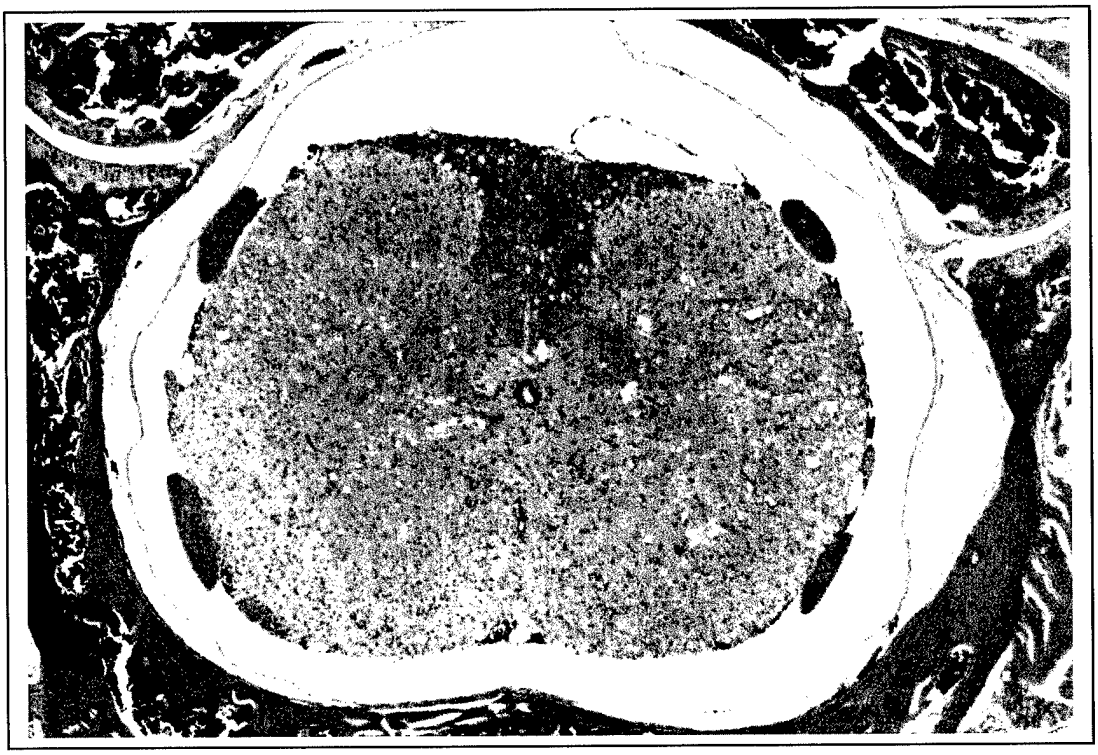

Figure 3.32: H+E staining of a serial section of an infected mouse treated with saline from 4-6 months post-infection. Magnification 40X. Notice the lack of darker staining of the white matter.

3.22 Electron microscopy

Mice infected for 6 months were sacrificed and their spinal cords were prepared for evaluation via electron microscopy as previously described in order to characterize demyelinating, inflammatory, and status spongiosus lesions in TMEV. Evidence of beginning demyelination is illustrated in figure 3.33 where the lamellae of the myelin sheath can be seen separating around an axon in the lower right quadrant. In figure 3.34, there is marked disorganization of an axon in the spinal cord of a mouse that has been infected with TMEV. There is also splitting of the myelin sheath that could possibly be eliminated as artifact. Status spongiosis and demyelination is shown in figure 3.35 where there are large spaces seen between myelinated axons. Lastly, figure 3.36 illustrates the accumulation of lymphocytes in the meninges of a TMEV infected mouse at 6 months post-infection.

Discussion

The post-mortum histological analysis of lesions in the central nervous system of 83 patients with MS has led to the suggestion that two different patterns of lesion development exist (Lucchinetti et al., 2000). The two patterns consist of T-cell-mediated or T-cell plus antibody-mediated autoimmune encephalomyelitis or primary oligodendrocyte damage caused by a viral or bacterial infection either of which results in demyelination (Lucchinetti et al., 2000). To further support the theory that T-cell-mediated and T-cell plus antibody-mediated autoimmune encephalomyelitis as a cause for lesion development, it has been reported that MS patients have T-cells in the CNS that produce the Th1 proinflammatory cytokines during relapsing episodes (Correale et al.,

1995; Voskuhl et al., 1993; Olsson et al., 1990) and anti-inflammatory Th2 cytokines during remission phases (Correale et al., 1995).

EAE and Theiler's viral infection, two animal models for multiple sclerosis, have similar pathologies (Dal Canto et al., 1995). Knowing that IFN-τ has proven to be an effective treatment in EAE by preventing acute induction and superantigen reactivation of EAE via suppressor cells (Soos et al., 1995; Majtaba et al., 1997), it was of interest to investigate the effect of IFN-τ on lesion development in the CNS.

It is interesting to note that the data generated during the present study supports the findings of interferon-tau treatment of EAE mice. As expected, a decrease in inflammation, status spongiosus, and demyelination was observed in the H+E and immunostained slides of the spinal cords of SJL/J mice when interferon-tau was administered from 4-6 months post-infection which is during the chronic phase of the TMEV infection. The remyelination seen in the MOG slides has also been seen in EAE where inflammation appears to enhance migration of oligodendrocytes though remyelinated myelin never regains its normal thickness after a demyelinating lesion (Ludwin, 1997; Tourbah et al., 1997). The decrease in lesion presentation was attributed to the down regulation of proinflammatory Th1 cytokines via interferon-tau administration in EAE. The current hypothesis is that IFN-τ protection in EAE occurs via Th2 cytokine activation as noted with increased IL-10 production (Soos et al., 1997), the inhibition of B-cell and T-cell responses in active and chronic EAE (Majtaba et al, 1998), and the lack of inflammatory lymphocytic lesions in the CNS (Soos et al., 1997) during IFN-τ treatment.

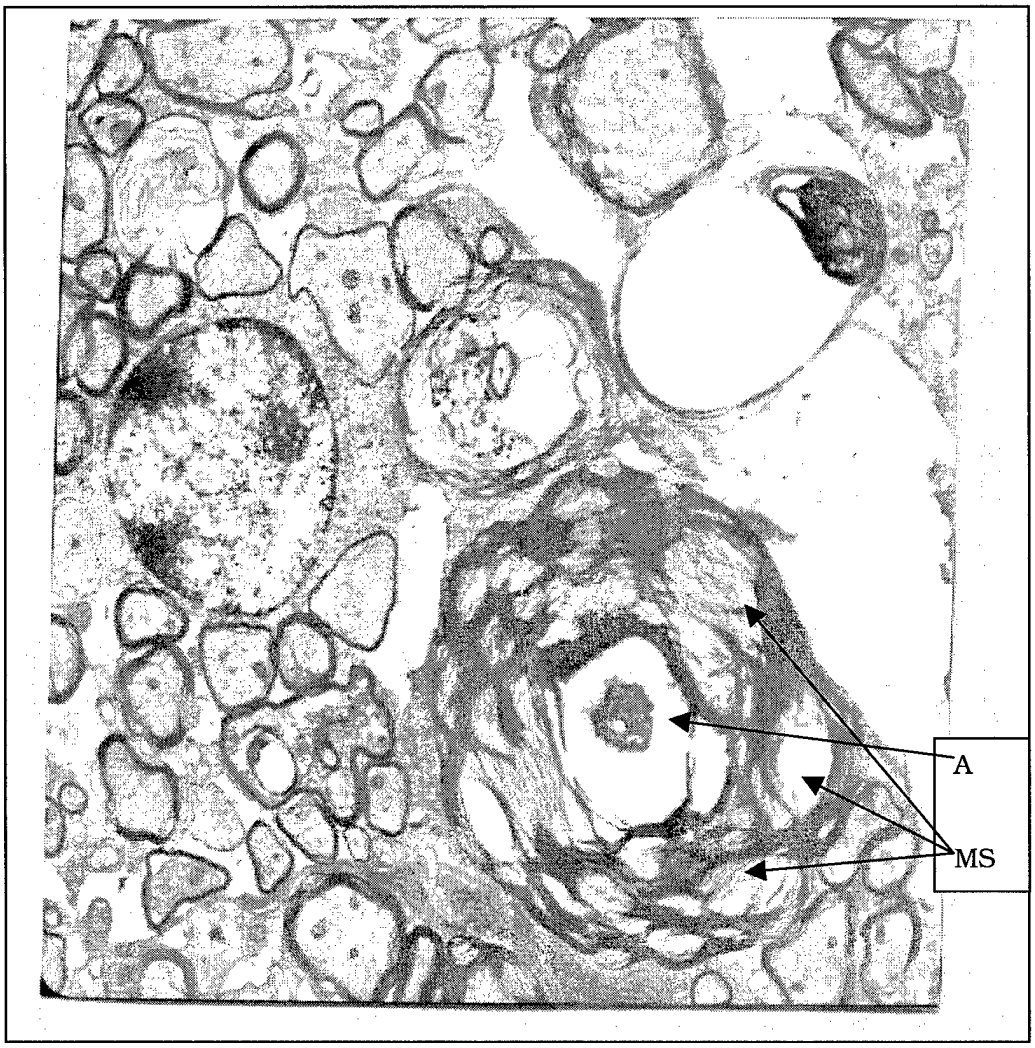

Figure 3.33: EM of demyelination in the ventral funiculi. Magnification 28,000X. Electron microscopy picture of a TMEV demyelinating lesion from the ventral funiculi of a mouse spinal cord 6 months post-infection. Note the separation of the lamellae of the myelin sheath surrounding the axon in the lower right quadrant and the increased interstitial space condusive of status spongiosus in the upper right quadrant of the photograph. A = axon, MS = myelin sheath

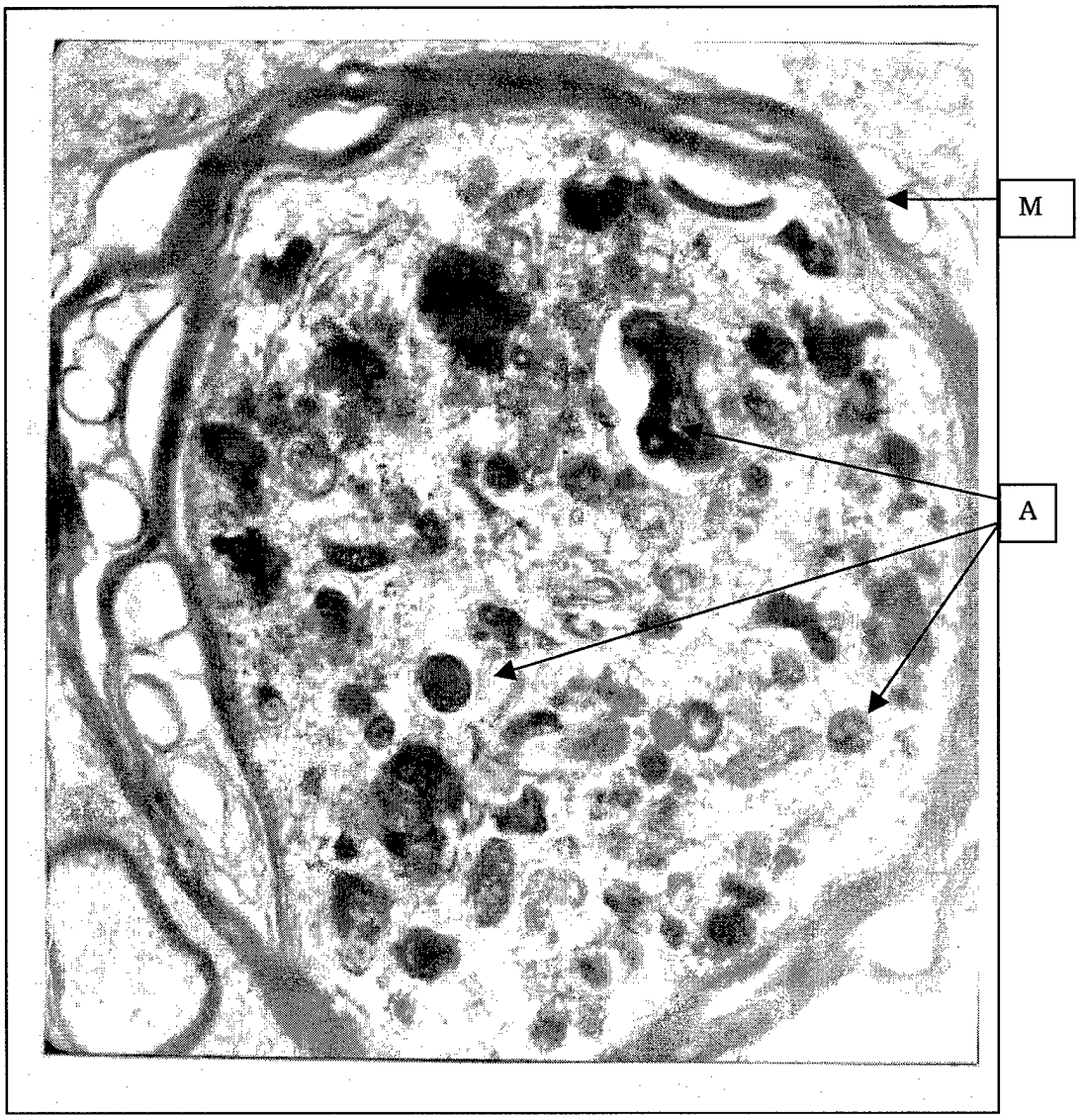

Figure 3.34: EM of demyelination in the ventral funiculi. Magnification 40,000X. Electron microscopy picture of a TMEV demyelinating lesion from the ventral funiculus of a mouse spinal cord 6 months post-infection. Note the myelin unwrapping around the axon and the organelle damage in the neuron cell body. A = axon, M = myelin

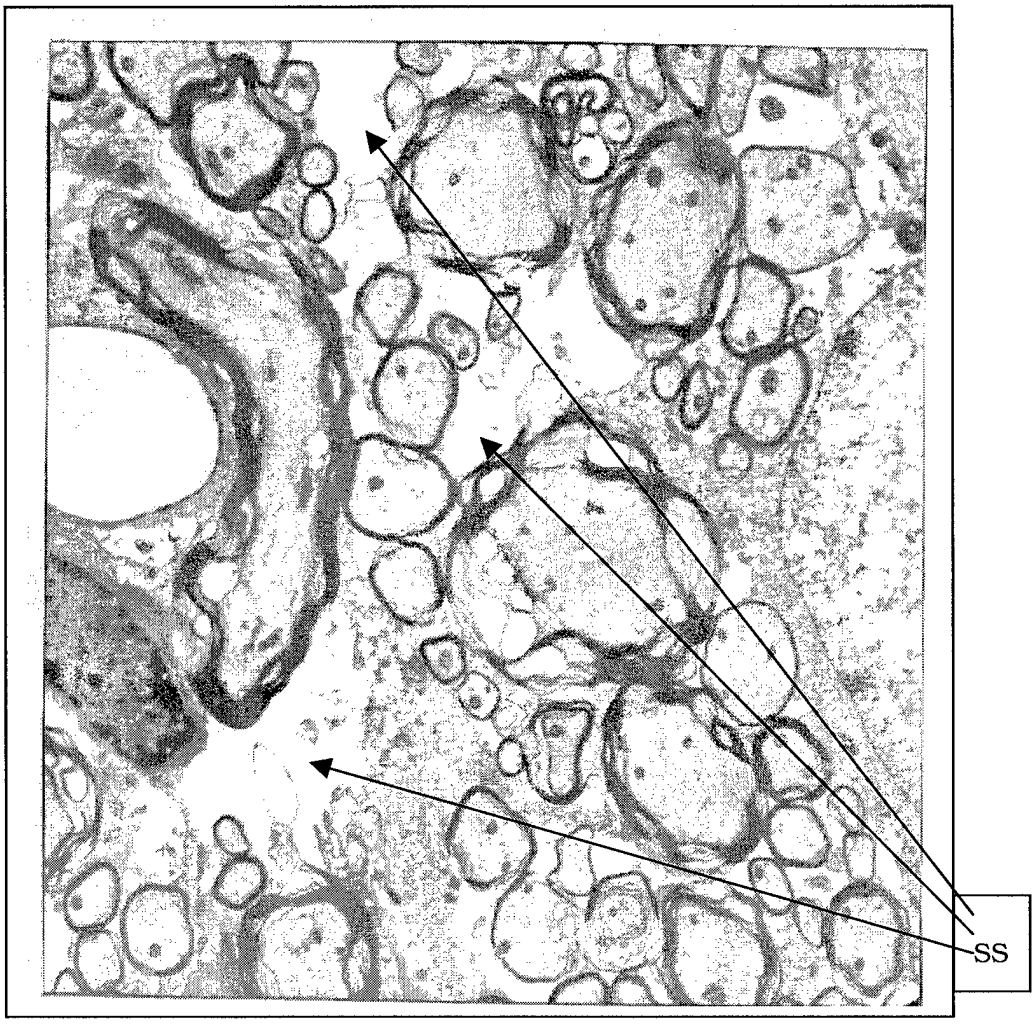

Figure 3.35: EM of demyelination and status spongiosus in the ventral funiculi. Magnification 28,000X. Electron microscopy picture of a TMEV demyelinating lesion illustrating status spongiosus in the ventral funiculus of a mouse spinal cord 6 months post-infection. The splitting of the myelin sheath around the central axon is suggestive of early demyelination, but could not be totally eliminated as being the result of artifact. SS = status spongiosus

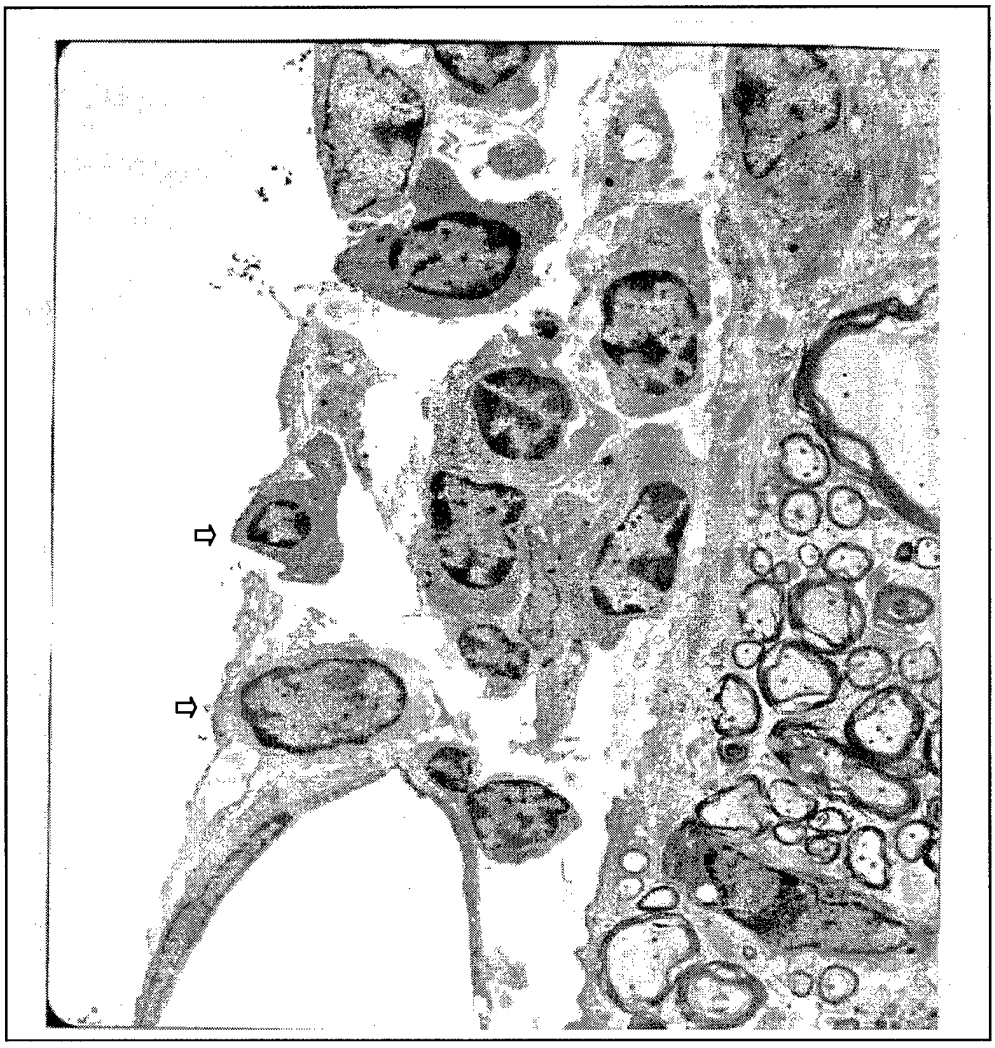

Figure 3.36: EM of meningeal inflammation. Magnification 28,000X. Electron microscopy picture of a Theiler's virus inflammatory lesion from the menegies of a mouse spinal cord 6 months post-infection. Note the lymphocytes present in the left half of the picture.

Interferon-tau treatment was not beneficial in TMEV when administered before viral infection, as seen in the H+E staining of the spinal cords of mice pretreated with interferon-tau before viral infection. Th1 proinflammatory cytokines including interferon-gamma are needed for the immune system to mount an anti-viral response and decrease viral titers (Rodriguez et al., 1995). It follows then that the administration of an immunomodulator like interferon-tau that is able to decrease Th1 cytokine production would result in an increase in lesion presentation due to viral infection when given before the virus.

The stage of disease progression during which interferon-tau treatment occurs is very important to the effectiveness of treatment as seen with the 2-6 month treatment group where no significant difference was observed between treated and infected controls. It is believed this is due to the change of cytokine expression during different stages in TMEV. Like MS, there appears to be a difference in cytokine expression during the chronic demyelinating phase of TMEV and the initial anti-viral phase of TMEV. It is important that interferon-tau is given during the chronic demyelinating phase to suppress the production of autoimmune damage to the CNS by Th1 cytokine expression for the most significant improvement to occur. It is possible that tolerance to interferon-tau may develop if administration continued past 2 months even during the chronic phase of TMEV. Further investigation into the effectiveness of long-term treatment is necessary.

Another interesting factor should be discussed concerning the immunostained slides of the 4-6 month IFN-τ treated mice. MOG staining was present in the gray matter of IFN-τ 4-6 month infected treated and non-treated, infected mice that was not present in the

healthy controls. Two main possibilities have been proposed to explain this phenomenon; 1) the MOG antibody used is staining pieces of myelin that have been processed by antigen presenting cells and is now being expressed on the outside of those cells in the gray matter or 2) administering Theiler's virus before the CNS was fully myelinated, interrupted normal migration of oligodendrocytes to the white matter of the spinal cord and the satellite cells were stained. Double staining with MHC antibodies is planned for the future to answer some of these questions.

It has previously been reported that IFN-τ is able to induce hepatocyte apoptosis in sheep when 5.2×10^6 antiviral doses are administered subcutaneously twice per day. The livers of these sheep were examined after 1-3 days of IFN-τ treatment (Kim et al., 2000). No lesions were detected via light microscopy and H+E staining in the livers of the mice in this study that were treated from 4-6 months post-infection with IFN-τ. This indicates that IFN-τ is not hepatoxic at 10^5 antiviral units in the TMEV animal model of MS when given i.p. once daily.

IV. IMMUNOLOGICAL ANALYSIS

Introduction

4.1 Theiler's virus and EAE

Theiler's murine encephalomyelitis virus (TMEV) and experimental allergic encephalomyelitis (EAE) are two murine models that are used to investigate multiple sclerosis in the laboratory setting. TMEV is primarily a model for the viral etiology of MS, whereas EAE focuses on the autoimmune component.

TMEV is a picornavirus that was first characterized as a murine model for MS in 1975 when it was discovered that TMEV infection causes an inflammatory demyelinating disease (Lipton, 1975). TMEV strains have been divided into two subgroups composed of the neurovirulent GDVII and FA substrains and the less virulent TO substrains. The TO substrains include BeAn 8386, DA, WW, TO_4 and Yale (Lipton, 1980). Intracranial (i.c.) infection of susceptible mice with neurovirulent GDVII results in acute fatal encephalitis during the first week of infection. Alternatively, permissive hosts infected intracranially with one of the TO substrains of TMEV develop a flaccid paralysis by 2 weeks post-infection (p.i.) by which time viral antigens and genomic RNA are located in the neurons of the gray matter. The chronic persistence of TMEV in the white matter of the spinal cord begins one month p.i. and continues throughout the duration of the animal's life (Yamada et al., 1991).

EAE is induced by the injection of myelin basic protein (Meyer et al., 1996) or proteolipid protein plus adjuvant into laboratory animals including mice (Ke and Kapp, 1996). EAE can also develop through

adoptive transfer of autoreactive T-cells to naïve mice which establishes it as an autoimmune model for MS (McCarron and McFarlin, 1988; Satoh et al., 1988; Kuchroo et al., 1992).

EAE mimics MS in that it is an autoimmune inflammatory disease of the CNS that is T-cell-mediated (Zamvil and Steinman, 1990). The upregulation of IL-2, IFN-γ, and TNF-β (Van der Veen and Stohlman, 1993; Cua et al., 1995; Olsson, 1995) and the down regulation of TGF-β, IL-10, and IL-4 (Okuda et al., 1995; Issazadeh et al., 1996; Diab et al., 1997) have been detected in the CNS of animals with EAE resulting in the conclusion that development of disease is the result of Th1 cell cytokine secretion and the balance between Th1/Th2 cell activation (Van der Veen and Stohlman, 1993; Cua et al., 1995; Olsson, 1995).

4.2 DTH and Theiler's virus

Type IV delayed-type hypersensitivity is a form of cell-mediated immunity that occurs 24-48 hours after challenge with an antigen previously encountered by the host. This is a T-cell mediated response resulting in the infiltration of macrophages and inflammation. In the case of a Theiler's virus infection, the major protein of concern when the focus is autoimmunity is host myelin basic protein. Myelin that was prepared from the brains of adult SJL mice initiates a DTH reaction within 24 hours when injected into the ears of SJL mice chronically infected with TMEV strain BeAn. The intensity of the DTH reaction is assessed via ear thickness where PBS injections into the opposite ears of the same mice are used as controls (Borrow et al., 1998). Viral epitope spreading also results in a DTH reaction in the Theiler's model as TMEV induces new T-cell responses during the course of infection (Miller et al., 1997). Myelin oligodendrocyte glycoprotein (MOG) and myelin

proteolipid protein (PLP) are both suggested autoantigens in MS because they are able to initiate a DTH reaction in mice infected with TMEV (Slavin et al., 1998; Wang et al., 1999).

During the course of chronic TMEV infection, Th1 cells specific to TMEV are reported to be the direct cause of a delayed-type hypersensitivity reaction that occurs and contributes to bystander demyelination (Miller and Gerety, 1990). DTH responses may also be used to determine the immune response to TMEV using UV-irradiated whole virus particles (Pullen et al., 1994; Lipton et al., 1995; Drescher et al., 1998; Murray et al., 1998; Koh et al., 2000), active TMEV (Karpus et al., 1995; Inoue et al., 1996; Inoue et al., 1997; Inoue et al., 1998; Inoue et al., 1999), or specific viral peptides (Miller et al., 1995; Burt et al., 1999; Pavelko et al., 2000). In all of these experiments, the DTH response was used as an assessment of the efficiency of the immune system to recognize virus after either an immunomodulating treatment or a genetic mutation that affects MHC presentation. Therefore, it is widely accepted that DTH responses to myelin, MOG, PLP, and TMEV are involved in the pathogenesis of Theiler's virus-induced demyelinating disease.

4.3 Cytokine expression in the CNS during chronic EAE or TMEV

It is important to characterize the expression of Th1 and Th2 cytokines in the CNS during EAE or TMEV infection since it is believed the change in cytokine expression is partially responsible for the induction of demyelination seen in both animal models. During the exacerbation phase of EAE in SJL/J mice, 14 days post-inoculation, Th1 cytokines IL-2 and IFN-γ are up-regulated in the brain, spinal cord, and cerebral spinal fluid (Renno et al., 1994). In contrast, Th2 cytokines are induced during remissions.

The cytokine data concerning chronic TMEV infection is controversial. Using the DA strain of TMEV, at 40 days p.i. in SJL/J mice, Sato et al. using RNA protection assays found that the Th1 cytokines IL-1 and IL-2 were not detectable in the spinal cord whereas Th2 cytokines, IL-10, and Th1 cytokines TNF-α, IL-12, and IFN-γ were elevated when compared to controls. In the brains of the same animals, IL-10, IL-12, TNF-α, IL-1, IL-2, IL-6, and IFN-γ were not detected at 40 days p.i. but, IL-4 production was high in the brain (Sato et al., 1997). It has also been reported that IFN-γ, IL-1, IL-2, IL-6, IL-12, TNF-α, TGF-β1, IL-4, IL-5 and IL-10 cytokine RNA was detected at levels above controls at 60 days p.i. in SJL/J mice also infected with DA with real-time PCR analysis (Chang et al., 2000). Since there appears to be inconsistencies between 40 and 60 days p.i. with the same TMEV strain, it would be interesting to see if there was a difference in cytokine expression in the CNS of mice infected with the BeAn as compared to the DA strain of TMEV.

4.4 Interferon-tau

IFN-τ has been characterized as one of the pregnancy recognition signals that is secreted by sheep trophoblasts between days 9 and 24 of pregnancy (Godkin et al., 1984). It functions as an anti-luteolytic agent by decreasing the endometrial gene expression of oxytocin receptors (Vallet and Lemming, 1991; Spencer et al., 1996). IFN-τ also acts in an anti-inflammatory capacity *in vivo* by inhibiting Th1 lymphocyte proliferation (Newton et al., 1989, Niwano et al., 1989; Skopets et al., 1992; Assal-Meliani et al., 1993), promoting the production of placental Th2 cytokines IL-4 and IL-10 (Chaouat et al., 1995), and activating NK

cells in utero (Tuo et al, 1993). IFN-τ has 70% homology with IFN-α (Stewart et al., 1989), and is therefore classified as a type I interferon with anti-viral activity comparable or superior to IFN-α in the treatment of ovine lentivirus *in vivo* and *in vitro* (Juste et al., 2000), human papilloma virus *in vitro* (Johnson et al., 1999), and HIV *in vitro* (Dereuddre-Bosquet et al., 1996).

IFN-τ has proven to be an effective treatment in EAE by preventing acute induction and superantigen reactivation of EAE via suppressor cells (Soos et al., 1995; Majtaba et al., 1997). The current hypothesis is that this protection occurs via Th2 cytokine activation as noted with increased IL-10 production (Soos et al., 1997), the inhibition of B-cell and T-cell responses in active and chronic EAE (Majtaba et al, 1998), and the lack of inflammatory lymphocytic lesions in the CNS (Soos et al., 1997) during IFN-τ treatment.

Antibodies to IFN-τ were detected in SJL/J mice with EAE following i.p. treatment with IFN-τ. However, these antibodies are non-neutralizing (Soos et al., 1997) and therefore do not interfere with the specific activity of IFN-τ. Oral administration of IFN-τ under the same conditions does not lead to the production of anti-IFN-τ antibodies that are detectable via ELISA using serum from terminal cardiac bleeds (Soos et al., 1997).

As mentioned previously, administration of IFN-τ is able to increase the production of IL-10 in the serum of SJL/J mice that have EAE. The induction of IL-10 was noted in both oral and i.p. administration if treatment continued for more than 20 days (Soos et al., 1997). IL-10 is also increased in SJL/J mice with chronic EAE during oral IFN-α/β therapy when given over a seven week period (Brod et al., 1994).

4.5 Corticosterone and MS treatment

Corticosterone and cortisol are glucocorticoids that are secreted by the adrenal cortex. Both hormones are products of cholesterol catabolism and they differ by one hydroxyl group. Healthy humans normally produce a 1:5 ratio of corticosterone to cortisol whereas mice only produce corticosterone.

Multiple sclerosis patients are known to have an exaggerated cortisol release when relapsing-remitting MS progresses into secondary progressive MS. This is attributed to proinflammatory cytokines impairing normal glucocorticoid function and the increase in neuronal damage (Bergh et al., 1999). This theory is supported by the fact that neuroendrocine over-activation is seen in MS patients with acute CNS inflammation (Fassbender et al., 1998)

Glucocorticoid therapy can be beneficial in MS treatment, but effectiveness is dependent on the stage of disease progression. Treatment of MS patients with low-dose corticosteroids, 10-20 mg prednisolone daily, had no significant effect on MS progression with chronic progressive or relapsing MS (Tourtellotte and Haerer, 1965; Millar et al., 1967). In contrast, rapid clinical improvement is seen in MS patients in acute relapse who have been treated intravenously with methylprednisolone ranging from 250 mg daily to 20 mg/kg daily for 2-7 days (Dowling et al., 1980; Buckley et al., 1982; Abbruzzese et al., 1983; Goas et al., 1983; Newman et al., 1983; Barnes et al., 1985; Durelli et al., 1986; Milligan et al., 1987; Thompson et al., 1989).

The clinical benefits seen in patients with glucocorticoid therapy is attributed to the corticosterone immunosuppressive effects that are dosage dependent. Supraphysiological corticosteroid dosage can inhibit antibody-dependent cellular cytotoxicity (Nair and Schwartz, 1984),

neutrophil complement and IgG receptor binding (Forslid and Hed, 1982), antigen presentation by monocytes (Dumble et al., 1981; Hirschberg et al., 1982; Rhodes et al., 1986), 1984), astrocyte binding to measles-infected cells (Maki et al., 1980), monocyte cellular differentiation (Rinehart et al., 1982) and leukocyte production of IL-2 and IFN-γ (Cesario et al., 1986; Arya et al.).

IFN-τ may mediate its therapeutic effects in the TVID model by up regulating IL-10 production which would, in turn, decrease damaging DTH responses. IFN-τ has been reported to increase corticosterone levels in sheep (Ott et al., 1997). High levels of corticosterone would result in immunosuppression which would be beneficial to TMEV-infected mice during the chronic demyelinating phase of the disease. Therefore, corticosterone levels were also measured during TVID.

RNA protection assays on spinal cord tissue, DTH reactions to myelin, serum IL-10 and corticosterone levels, and antibody production to IFN-τ were measured to determine if IFN-τ is able suppress the Th1 response to TMEV chronic infection in female SJL/J mice.

Materials and Methods

4.6 Mice

3-week-old female SJL/j mice (Harlan, Houston, TX) were obtained and infected when 4-5 weeks old.

4.7 Virus

The BeAn 8386 strain of TMEV was a gift from Dr. Howard L. Lipton (Northwestern University, Evanston, IL). Virus was grown in BHK-21

cells, and the culture supernatant containing infectious virus was aliquoted and stored at -70°C before use. The viral titer was determined by plaque assay on BHK-21 cells (Rueckert and Pallansch, 1981).

4.8 Interferon-tau

The IFN-τ was a gift from Dr. Fuller W. Bazer (Texas A&M Health Science Center, College Station, TX). IFN-τ was isolated as previously described (Ott et al., 1991), diluted in 20mM Tris buffer pH 7.5 with 150mM NaCl, aliquoted and stored at -70°C before use. Protein concentration was determined by the Lowry assay (Lowry et al., 1951).

4.9 Infection of mice and assessment of clinical signs of demyelination

Mice were anesthetized with Metofane™ (Schering-Plough Animal Health, Union, NJ) and injected intracranially (i.c.) into the right cerebral hemisphere with 5 x 10^4 plaque forming units (PFU) of BeAn in a 20 uL volume.

After infection (day 0) all animals were examined weekly for the development of clinical signs indicative of demyelinating disease (Borrow et al., 1998). Clinical signs were scored on a scale from 0 to 6, 0 indicating a healthy animal and 1-6 representing gradual increasing severity of signs as follows: 1, piloerection and/or hunched posture; 2, piloerection and hunched posture plus unsteady gait; 3, very unsteady gait, weak grasp response when placed on wire grid, and occasional slight limb monoparesis; 4, severe hind limb weakness, incontinence, and weight loss; 5, paraparesis of hind limbs, severe weight loss, incontinence, dropped head; 6, moribund/dead.

4.10 IFN-τ treatment of mice prior to Theiler's virus infection up to the development of moderate demyelinating disease

Ten mice were injected with 10^5 units of IFN-τ intraperitoneally (i.p.) daily from 0 to 3 days pre-infection and 10 mice were treated with the same concentration of IFN-tau –3 to 60 days post-infection. Ten virus infected control mice for each treatment received 100uL of 0.9% saline i.p. during the same intervals. All perfusions occurred at 6 months post-infection (p.i.).

4.11 IFN-τ treatment of mice with moderate demyelinating disease

Ten mice were injected with 10^5 units of IFN-τ i.p. daily from 2 months to 6 months post-infection. Ten virus infected control mice received 100uL of 0.9% saline i.p. at the same intervals. All perfusions occurred at 6 months post-infection.

4.12 IFN-τ treatment of mice with severe demyelinating disease

Two experiments were performed to test the therapeutic potential of IFN-τ in mice suffering from severe demyelinating disease. In each experiment, 10 mice were injected with 10^5 units of IFN-τ (Soos et al., 1995) i.p. daily from 4 months to 6 months post-infection. Ten virus infected control mice received 100uL of 0.9% saline i.p. at the same intervals. All perfusions occurred at 6 months post-infection.

4.13 Methylprednisolone treatment of mice

Ten mice were injected i.p. with pulse injections of 1mg methylprednisolone (40mg/kg) (Pharmacia & Upjohn, Kalamazoo, MI) every three days from 5 months to 6 months p.i. to inhibit inflammatory responses (Pavelko et al., 1998). Ten virus infected control mice received 100uL of 0.9% saline i.p. at the same intervals. All perfusions occurred at 6 months post-infection.

4.14 IFN-τ treatment of non-infected control mice

Ten mice were treated from 5 to 7 months of age daily with 10^5 units of interferon-tau by i.p. injection. Ten non-infected control mice were injected with 100uL of 0.9% saline i.p. at the same time. The mice were perfused at 7 months of age.

4.15 White blood cell differential analysis of cardiac blood

Prior to the actual perfusion, approximately 1 ml of whole blood was collected via cardiac puncture and placed into 1.5 ml centrifuge tubes. The chest cavity of each animal was coated with heparin prior to collection. White blood cell differential analysis was performed in duplicate using a Celldyn 3500 automated cell counter (Abbott Laboratories, Abbott Park, IL). Results were reported as number of cells per milliliter of whole blood.

4.16 IFN-τ ELISA

Serum was collected via tail bleeding before and during IFN-τ treatment of all post-infection experimental groups. Serum samples were obtained from pretreated groups at 2 months post-infection. The serum was assayed for IFN-τ antibodies via ELISA as previously described (Johnson et al., 1998). Briefly, 96 well Immulon II flat bottom microtiter plates were treated with 0.2 ug/mL monoclonal antibody to IFN-τ (gift from Dr. Fuller Bazer, Department of Animal Science, Texas A&M University, College Station, TX) and incubated overnight at 4°C. After four washings with a 1:1 PBS-Tween 20 solution, the plates were washed with distilled water. 100uL of 1% BSA in carbonate coating buffer was added to each well and the plates were incubated as mentioned previously. The wash procedure was repeated. Duplicate serum samples as well as a positive control from a super-immunized mouse was added to the wells in systematic 1:20 dilutions. After a 40 minute incubation in 37°C with 4% CO_2, plate washing occurred and 50 uL of 1:10,000 dilution of horseradish peroxidase-conjugated rabbit anti-Mouse IgG (H+L) (Sigma, St. Louis, MO) was added to each well. Washing was repeated after a 60 min. incubation at 37°C in 4% CO_2. 50 uL ABTS in ABTS buffer (Boehringer Mannheim, Germany) was added for colormetric detection. The plates were read at 450nm after 50min incubation at room temperature (Emax precision microplate reader, Molecular Devices, CA).

4.17 IL-10 ELISA

Serum samples from IFN-τ treated and control mice were collected before and during IFN-τ treatment. The serum was analyzed via ELISA for IL-10 with the aid of a kit as per the manufacturer's instructions (R&D Systems Inc., Minneapolis, MN). All samples were analyzed in duplicate.

4.18 Myelin-specific DTH testing

DTH testing followed the procedure outlined in Borrow et al., 1998. Briefly, 10 chronically infected mice at 6 months pi were tested for DTH against myelin after eight weeks of IFN-τ treatment. Ten control infected mice at 6 months pi with saline treatment were also analyzed for DTH reactions by injecting the ears of the mice with myelin membrane and measuring the amount of ear thickness with calipers 24 and 48 hours after myelin injection. PBS was injected in the ears of the mice as a control.

4.19 Radioimmunoassay for corticosterone

Serum samples were analyzed in duplicate using ImmuChem™ Double Antibody Corticosterone RIA kit (ICN Biomedicals Inc., Costa Mesa, CA). Briefly, samples were diluted 1:200 in serial diluent and corticosterone-^{125}I was added to all the samples and controls. Corticosterone antibody was added to samples and internal controls from the kit and incubated at room temperature for 2 hours. The precipitant solution was then added, the tubes were vortexed for one minute, and centrifuged at 1000

x g for 15 minutes. The supernatant was removed and all tubes were counted on a gamma counter for corticosterone-specific radioactivity.

4.20 Preparation of tissue for RNase Protection Assay (RPA) analysis

At six months p.i., mice were anesthetized with Rompun (5mg/kg) (Bayer Corp., Shawnee Mission, Kansas) and Ketamine (100mg/kg) (Fort Dodge Animal Health, Fort Dodge, Iowa). Terminal cardiac bleeds were performed and spinal cord and brain tissue was removed. All tissues were preserved in cryovials, placed in liquid nitrogen for 24 hours, and stored at –80°C until RNA extraction. Tissue was homogenized and RNA extracted using a TRIZOL reagent kit (Life Technologies Inc., Rockville, MD) as described by manufacturer (Gilman, 1993).

4.21 RPA

The RPA was performed to compare the chemokine and cytokine profiles in the spinal cords of mice treated 4-6 months p.i. with IFN-τ or saline that were infected with Theiler's virus. Total RNA was amplified from the samples using the Direct Protect TM Lysate Ribonuclease Protection Assay (RPA) (Ambion, Austin, TX) according to the manufacturer's instructions. RPA multiprobe kits were obtained from Pharmingen (San Diego, CA) and used according to the manufacturer's instructions. Radiolabelled anti-sense RNA transcripts for cytokines and chemokines were generated using RiboQuant probe sets for: **MCK-2b** (IL-12p35, IL-12p40, IL-10, IL-1a, IL-1b, IL-1Ra, IGIF, IL-6, IFN-γ, MIF, L32, GAPDH)

Briefly, T7 polymerase synthesized transcripts were hybridized to 10mg of total RNA from brain and spinal cords for 16 hrs at 56°C. The non-hybridized RNA was then digested with RNase A/T1. Densitometric scanning (IS-1000 Digital Imaging System) was used to quantify transcript levels. After precipitation, RNA samples were analyzed by 5% denaturing urea polyacrylamide gel electrophoresis. The gels were exposed to a phosphorscreen. RNA loading estimations were conducted with phosphorimaging by comparison of intensity with RNA fragments from two housekeeping genes, L32 and GAPDH (Gilman, 1993).

4.22 Statistical analysis

A repeated measures analysis of covariance (ANCOVA) were performed on the DTH, IFN-τ ELISA, and IL-10 ELISA data. When appropriate, standard deviations were used to determine error bars and 95% confidence intervals.

Results

4.23 White blood cell differential analysis of cardiac blood

IFN-β treatment of MS patients has led to lymphopenia, neutropenia, and leukopenia (PRISMS study group, 1998) therefore circulating white blood cell counts were performed on the 4-6 month p.i. IFN-τ treated mice and their controls. No statistical difference of circulating white blood cell counts was found using ANCOVA analysis between BeAn infected mice treated with interferon-tau 4 to 6 months post-infection,

infected mice treated with saline 4 to 6 months post-infection, and non-infected mice with no treatment of the same age (Table 4.1) $p>0.05$. Therefore, IFN-τ treatment does not appear to cause the same side-effects with regard to changes in white blood cells as IFN-β treatment in MS patients.

Table 4.1: WBC analysis performed on terminal cardiac bleeds from SJL/J mice. Groups A and B were both infected with Theiler's virus. Group A received i.p. saline injections from 4-6 mo. p.i. Group B was given IFN-τ also from 4-6 mo. p.i. Group C was not infected with TMEV and received no treatment. All mice were perfused at 7 months of age. n=5 for each condition. No statistical difference was detected between all three conditions for all cell types ($p>0.05$).

Group	**A**	**B**	**C**
Infected	+	+	-
Treatment	Saline	IFN-tau	None
Neutrophils	19.819 ± 13.744	14.591 ± 3.026	9.367 ± 3.857
Lymphocytes	68.410 ± 17.868	73.600 ± 3.652	81.286 ± 6.240
Monocytes	8.165 ± 5.377	8.798 ± 3.696	6.106 ± 7.708
Eosinophils	2.804 ± 0.981	2.170 ± 0.708	2.651 ± 1.088
Basophils	0.821 ± 0.363	0.854 ± 0.263	0.589 ± 0.542
Total WBC %	5.807 ± 2.124	5.865 ± 2.765	6.759 ± 3.156

4.24 IFN-τ ELISA

Significant levels of antibodies to interferon-tau were detected in non-infected and infected mice treated with IFN-τ from 5-7 months of age and 4-6 months post-infection respectively $p<0.05$. At the time of

perfusion, however, levels of antibodies to interferon-tau were not statistically different from background levels in the infected mice treated –3 to 0 days post-infection with interferon-tau probably because the serum was collected 6 months after interferon-tau administration $p>0.05$ (Figure 4.1).

4.25 IL-10 ELISA

Circulating IL-10 levels were elevated from background in the infected mice treated with methylprednisolone 5 to 6 months post-infection or with interferon-tau 4 to 6 month post-infection $p<0.05$. This was attributed to the anti-inflammatory effects of methylprednisolone and interferon-tau which both function to down regulate Th1 cytokines and increase Th2 cytokine production (Figure 4.2).

4.26 Myelin-specific DTH testing

The delayed-type hypersensitivity to myelin was greatly reduced in infected mice that received interferon-tau 4 to 6 months post-infection as opposed to infected, saline controls $p<0.05$. These results correlate with a decrease in the Th1 response of the interferon-tau treated mice (Figure 4.3).

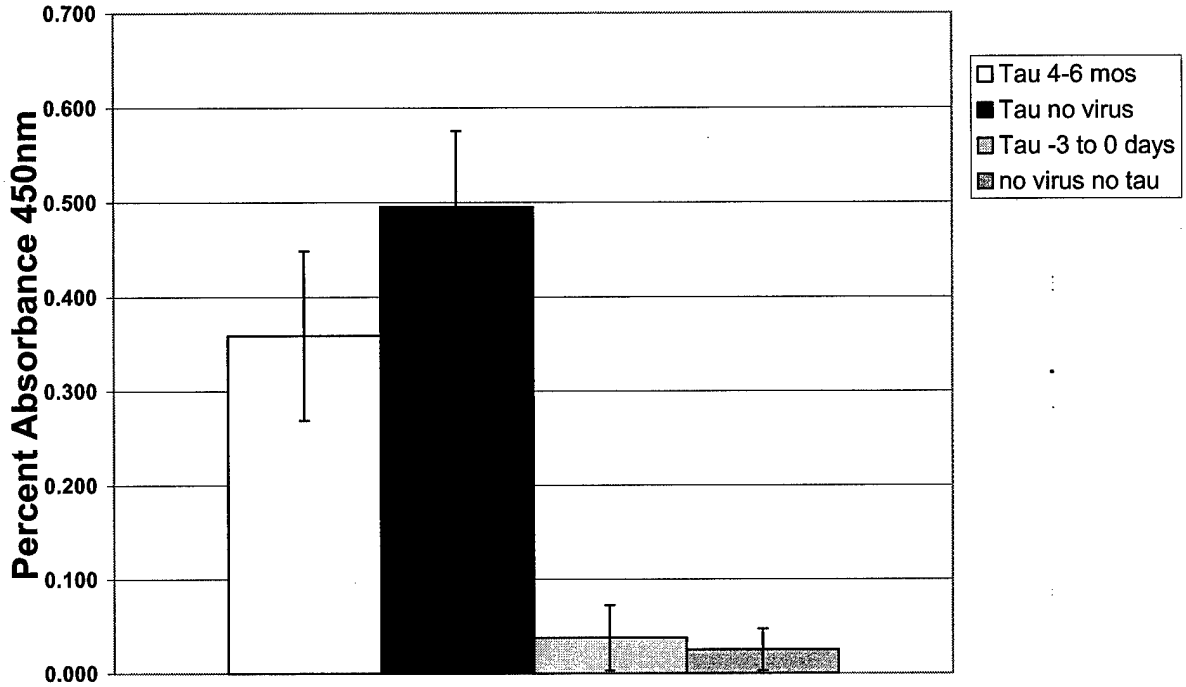

Figure 4.1: IFN-τ ELISA graph of all groups with IFN-τ treatment. IFN-τ ELISA performed on terminal bleeds at 7 months of age for all treatment groups to determine the antibody level to IFN-τ. The interferon-tau 4 to 6 months and interferon-tau –3 to 0 days were both infected with BeAn. Analysis was performed in duplicate. n=5 for each condition. The both infected and non-infected mice treated with IFN-τ from 4 to 6 months were significantly different than the control mice ($p<0.05$).

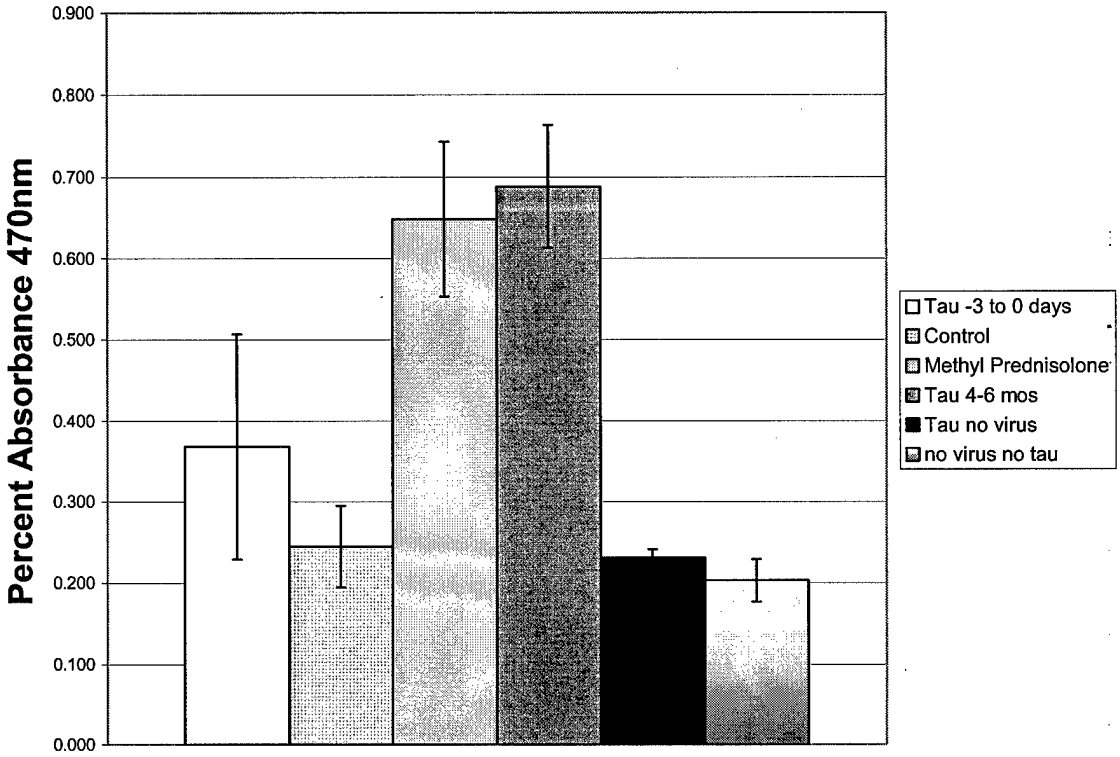

Figure 4.2: IL-10 ELISA graph of all treatment groups. IL-10 ELISA performed on terminal bleeds at 7 months of age for all treatment groups. The interferon-tau 4 to 6 months, interferon-tau –3 to 0 days, control, and methylprednisolone groups were all infected with BeAn. The control mice received saline 4 to 6 months post-infection. The interferon-tau, uninfected group received interferon-tau at 4 to 6 months post-infection. Analysis was performed in duplicate. n=5 for each condition. The mice treated with methylprednisolone and those treated with IFN-τ from 4 to 6 months p.i. were significantly different than the other treatment groups and control mice ($p<0.05$).

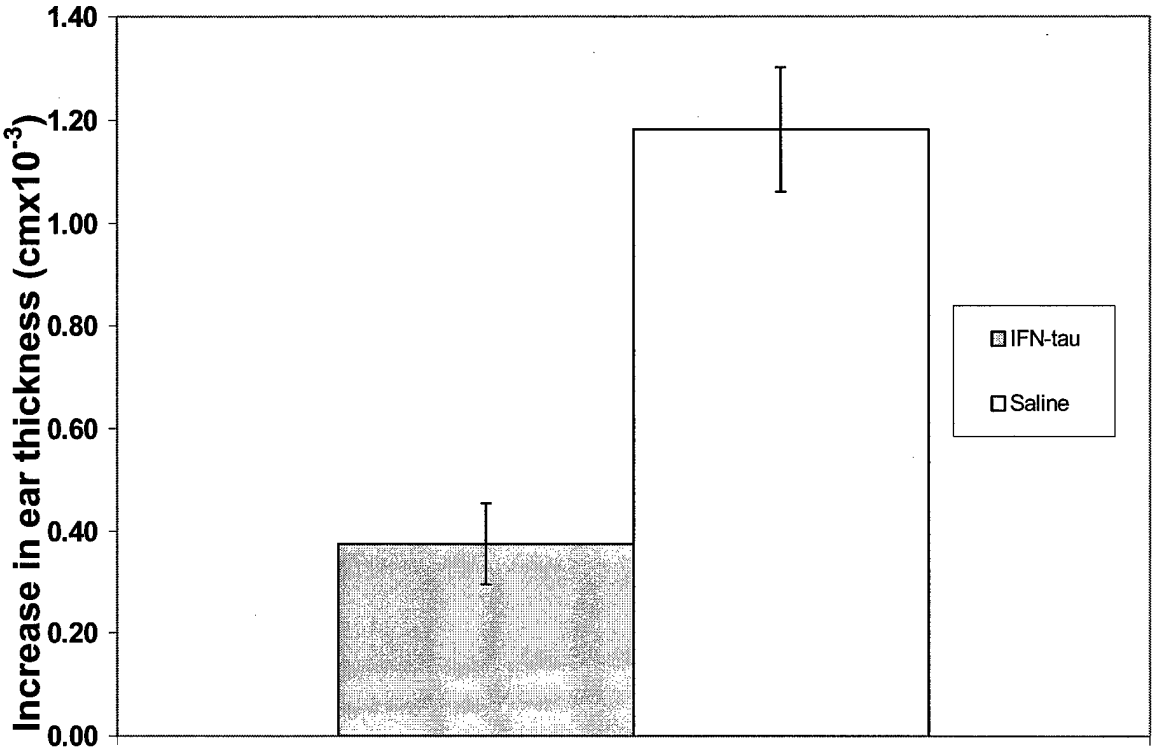

Figure 4.3: DTH graph of 4 to 6 months p.i. IFN-τ treatment and saline infected controls. Delayed-type hypersensitivity to myelin performed at 6 months post-infection 48 hours before perfusion. Both groups were infected with BeAn. The interferon-tau group received interferon-tau 4 to 6 months post-infection. All data was collected 24 hours after myelin injection and PBS control thickness measurements were subtracted from the myelin measurements. n=10 for both groups ($p<0.05$).

4.27 Radioimmunoassay of corticosterone

IFN-τ has been reported to elevate corticosterone levels in sheep (Ott et al., 1997). Therefore, corticosterone was measured in all experimental groups. IFN-τ treatment did not elevate corticosterone levels in non-infected mice (range 35-90 ng/ml). The RIA for corticosterone is considered preliminary data as the non-infected, 4 to 6 month interferon-tau treated group had an n of 2 (Table 4.2). However, a slight correlation ($R^2 = 0.7389$) was seen between corticosterone levels and the clinical scores of the mice at the time of serum collection (Figure 4.4). This correlation may increase with a higher sample number.

4.28 RPA

The RPA analysis of the spinal cord tissue extracted from infected mice treated with saline or interferon-tau 4 to 6 months post-infection resulted in a marked difference in cytokine expression between the two groups. Mice treated with interferon-τ showed an increase in IL-1, IL-6, IL-10 and IFN-γ compared to saline treated mice (Figures 4.5 and 4.6).

Table 4.2: Corticosterone v. clinical scores of all treatment groups. Corticosterone RIA on mouse serum samples in relation to clinical scores at the time of perfusion. All samples analyzed in duplicate. No relation was found between IFN-τ treatment and corticosterone levels.

Infected	Treatment	Timing of Treatment	Corticosterone ng/mL	Clinical Scores
+	IFN-tau	-3 to 0 days pi	216.97	3
+	IFN-tau	-3 to 0 days pi	885.32	4.5
+	IFN-tau	-3 to 0 days pi	659.8	4
+	IFN-tau	-3 to 0 days pi	430.11	3.5
+	IFN-tau	-3 to 0 days pi	724.73	4.5
+	Saline	4 to 6 months p.i	68.38	2
+	Saline	4 to 6 months p.i	300.1	3.5
+	Saline	4 to 6 months p.i	460.48	4
+	Saline	4 to 6 months p.i	48.79	3
+	Methylprednisolone	5 to 6 months p.i.	19.61	2.5
+	Methylprednisolone	5 to 6 months p.i.	354.18	3.5
+	Methylprednisolone	5 to 6 months p.i.	1000	5
+	Methylprednisolone	5 to 6 months p.i.	348.48	3.5
+	Methylprednisolone	5 to 6 months p.i.	510.14	3
+	IFN-tau	4 to 6 months p.i	124.93	2
+	IFN-tau	4 to 6 months p.i	445.95	3
+	IFN-tau	4 to 6 months p.i	885.32	3
+	IFN-tau	4 to 6 months p.i	498.4	2.5
-	IFN-tau	4 to 6 months p.i	90.62	0
-	IFN-tau	4 to 6 months p.i	35.26	0
-	None	None	51.84	0
-	None	None	12.66	0
-	None	None	115.03	0
-	None	None	214.22	0

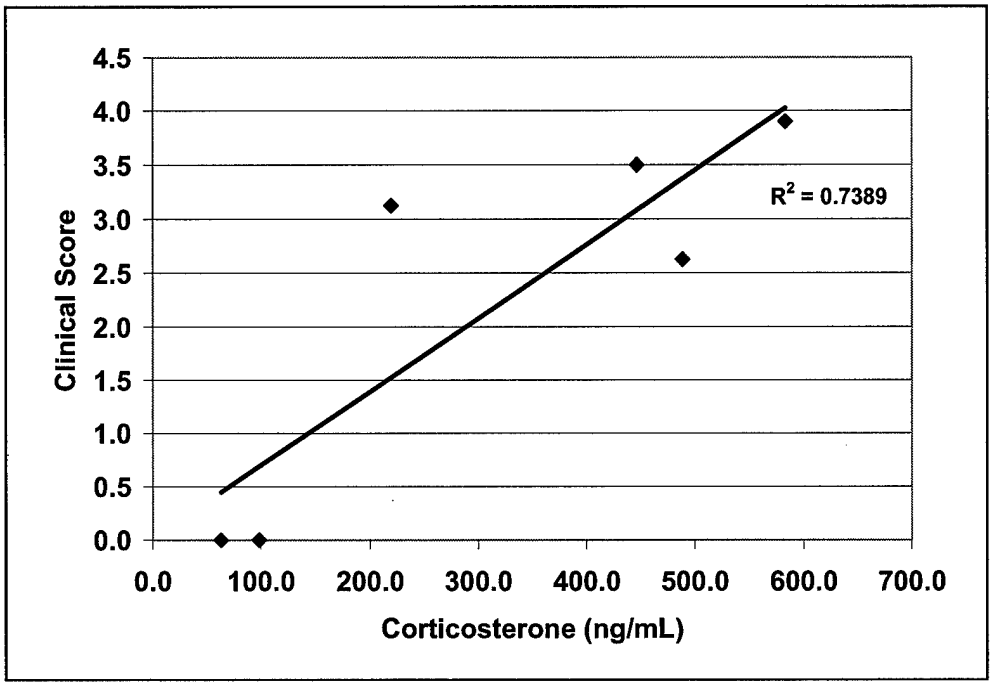

Figure 4.4: Preliminary correlation graph of corticosterone v. clinical scores. Corticosterone levels in serum v. clinical score at the time of perfusion. All samples were performed in duplicate. A slight correlation was found between corticosterone levels in serum and clinical scores of infected mice.

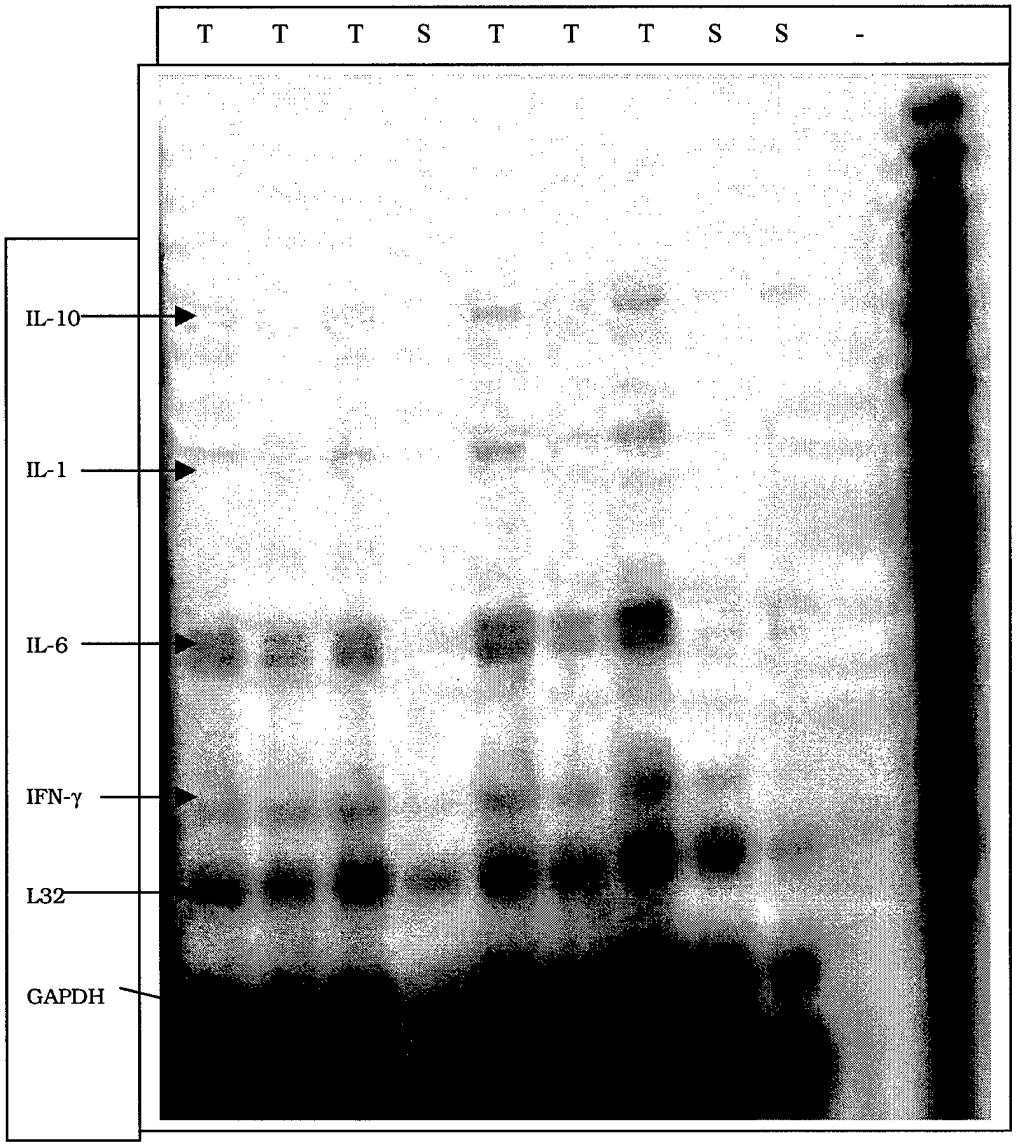

Figure 4.5: Spinal cord RPA mck-2b assay. Spinal cords from infected mice treated either 4 to 6 months with saline or interferon-tau were removed, the RNA extracted, and analyzed for cytokine RNA as outlined in methods. T = IFN-τ treated mice. S= saline treated mice. Note the increase in IL-6, IFN-γ, IL-1 and IL-10 in the mice treated with IFN-τ.

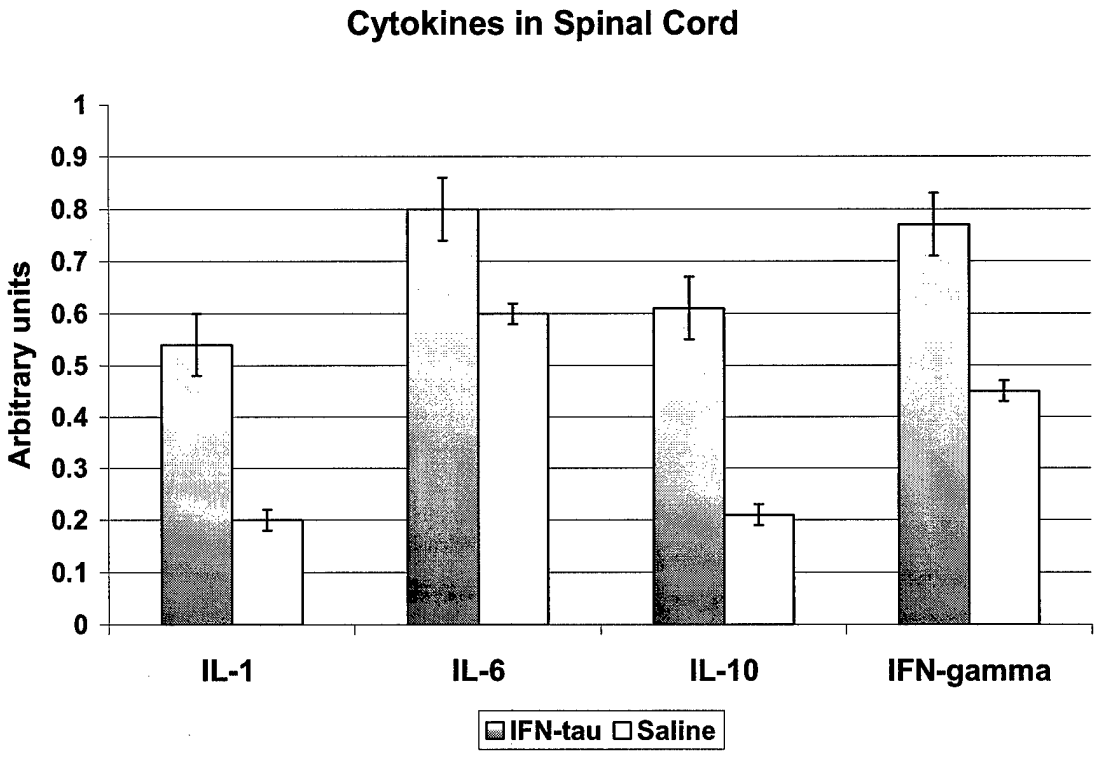

Figure 4.6: RPA analysis graph of cytokines expressed in infected IFN-τ treated mice and saline controls. Intensity was measured via phosphoimager. Housekeeping genes were used in comparison to cytokine intensity for all calculations. n=3.

Discussion

IFN-τ treatment of Theiler's virus-infected mice during late disease results in reduced inflammation and demyelination. When used to treat chronic BeAn TMEV infections, IFN-τ does not significantly change the white blood cell differential of female SJL/J mice when compared to non-treated controls. Occasionally, lymphopenia, neutropenia, and leukopenia are detected in MS patients as a result of IFN-β administration (PRISMS study group, 1998). This data confirms that IFN-τ does not effect circulating WBC counts as IFN-β does.

Due to the fact that ovine IFN-τ is a foreign protein in the murine system, it is expected that antibodies would be produced in the murine model. An antibody response to IFN-τ was detected, but similar findings from Soos et al. in the same strain of female mice found that these antibodies were non-neutralizing, therefore, they do not effect the activity of IFN-τ in vivo (Soos et al., 1997).

Reports that IFN-τ can inhibit Th1 cytokine production *in vivo* (Newton et al., 1989, Niwano et al., 1989; Skopets et al., 1992; Assal-Meliani et al., 1993) and upregulate the expression of Th2 cytokines (Chaouat et al., 1995) is further supported by the fact that an increase in IL-10 production is found when IFN-τ is used to treat chronic EAE (Soos et al., 1997) and chronic TMEV infection in SJL/J mice. The increase of IL-10 seen with IFN-τ administration in EAE is also detected to the same degree when IFN-α/β is given orally to treat EAE over a seven week period (Brod et al., 1994) suggesting that IFN-τ functions in a comparable manner to IFN-α/β in the treatment of autoimmune-induced MS like disease.

It is known from previous works that IFN-τ has anti-viral (Dereuddre-Bosquet et al., 1996; Johnson et al., 1999; Juste et al., 2000) and immunomodulating properties (Newton et al., 1989, Niwano et al., 1989; Skopets et al., 1992; Assal-Meliani et al., 1993; Tuo et al, 1993; Chaouat et al., 1995). A delayed-type hypersensitivity reaction to myelin occurs in TMEV-induced demyelinating disease and is proposed to contribute to bystander demyelination during chronic infection (Miller and Gerety, 1990; Miller 1997; Borrow et al., 1998). The data presented here suggest that IFN-τ treatment of chronically infected mice is able to decrease the Th1-induced DTH reaction to myelin that occurs during TMEV infections.

IFN-τ has been reported to increase serum corticosterone levels when administered to sheep (Ott et al., 1997). Increased corticosterone could result in immunosuppression, therefore, corticosterone was measured in the experimental mice. IFN-τ administration alone did not increase corticosterone levels. Interestingly, high levels of corticosterone were detected in virus infected mice with high clinical scores regardless of treatment group. No statistical correlation was detected between IFN-τ treatment and high corticosterone levels $p > 0.05$. Based on the preliminary data in this report, it is suspected that the level of circulating corticosterone in chronically TMEV-infected mice may correlate the level of clinical disease progression. Though not much is known about corticosterone and TMEV, previous works show that proinflammatory cytokines can impair normal glucocorticoid function in humans and contribute to neuronal damage in MS patients (Fassbender et al., 1998; Bergh et al., 1999). It stands to reason then that an increase in corticosterone would be seen during the chronic phase of TMEV when Th1 cytokine expression is elevated (Chang et al., 2000).

It is curious to note that the highest level of corticosterone reported in this study occurred in the methylprednisolone treated group. It is possible that the dose given was too low to significantly impair the Th1 cytokine damage in the TMVE chronic progressive MS model as seen in MS patients (Tourtellotte and Haerer, 1965; Millar et al., 1967). A larger sample size is needed to determine if there is a true correlation between serum corticosterone levels and TMEV clinical presentation.

The reports on cytokine mRNA in the CNS tissue of mice infected with TMEV are conflicting due to the fact that different investigators use different assays and perform their experiments during different times of infection. Using the DA strain of TMEV, at 40 days p.i. in SJL/J mice, Sato et al. found that the Th1 cytokines IL-1 and IL-2 were not detectable in the spinal cord whereas Th2 cytokines, IL-10, and Th1 cytokines TNF-α, IL-12, and IFN-γ were elevated when compared to controls. In the brains of the same animals, IL-10, IL-12, TNF-α, IL-1, IL-2, IL-6, and IFN-γ were not detected at 40 days p.i. but, IL-4 production was high in the brain (Sato et al., 1997). It has also been reported that IFN-γ, IL-1, IL-2, IL-6, IL-12, TNF-α, TGF-β1, IL-4, IL-5 and IL-10 cytokine RNA was detected at levels above controls at 60 days p.i. in SJL/J mice also infected with DA (Chang et al., 2000). Since there appears to be inconsistencies between 40 and 60 days p.i. with the same TMEV strain, it would be interesting to see if there was a difference in cytokine expression in the CNS of mice infected with the BeAn as compared to the DA strain of TMEV.

The RNA protection assay analysis reported here was performed on spinal cord tissue of mice with the BeAn strain of TMEV at 180 days p.i. (Figure 4.5). An increase was noted in IL-1, IL-6, IL-10, and IFN-γ in IFN-τ 4-6 month p.i. treated mice as compared to TMEV-infected

controls. The upregulation of IL-10 was expected due to the IL-10 serum ELISA data discussed previously. The increase in IL-1, IL-6, and IFN-γ maybe normal in TMEV BeAn infection but the control, infected mice did not express these cytokines at the same level as the IFN-τ treated mice. Though clinical presentation improved significantly with IFN-τ administration, the mice still have significant neurological impairment. This may be due to the fact that TMEV-infected mice develop truncated axons that can not be repaired via remyelination (McGavern et al., 2000). It is also possible that the activation of macrophages through IL-1 and IFN-γ and the production of antibodies through IL-6 expression contributes to the demyelination seen in the IFN-τ 4-6 month treated mice. Further cytokine analysis needs to be done on non-infected mice treated with IFN-τ and larger sample sizes to determine if the upregulation of the Th1 cytokines seen is due to the IFN-τ treatment or TMEV infection.

In conclusion, the increase in serum and spinal cord IL-10, decrease in DTH response to myelin, decrease in inflammation, and decrease in demyelination in the 4-6 month IFN-τ treated group imply that IFN-τ functions as a Th1 immunomodulator when administered during chronic TMEV infection. Viral RNA in the CNS needs to be measured as well as *in situ* hybridization cytokine analysis performed to determine if IFN-τ administration halts disease progression via anti-viral mechanisms as well as immunosuppressive means. IFN-α/β administration in TMEV-infected SJL/J mice did not decrease viral load so an anti-viral mechanism is questionable (Njenga et al., 2000).

V. Conclusions

Multiple sclerosis is a debilitating disease that effects the central nervous system of over 300,000 people in the United States. It can result in white matter demyelination, chronic inflammatory lesions, axonal damage, and deterioration of neurological function (Prineas and Raine, 1976; Prineas and Wright, 1978; Adams et al., 1989, Trapp et al., 1998).

The post-mortum histological analysis of lesions in the central nervous system of MS patients has led to the suggestion that two different patterns of lesion development exist. The two patterns consist of T-cell-mediated or T-cell plus antibody-mediated autoimmune encephalomyelitis and primary oligodendrocyte damage that is caused by a viral or bacterial infection and results in demyelination (Lucchinetti et al., 2000). To further support the theory that T-cell-mediated and T-cell plus antibody-mediated autoimmune encephalomyelitis as a cause for lesion development, it has been reported that MS patients have T-cells in the CNS that produce the Th1 proinflammatory cytokines during relapsing episodes (Correale et al., 1995; Voskuhl et al., 1993; Olsson et al., 1990) and anti-inflammatory Th2 cytokines during remission phases (Correale et al., 1995).

Since MS is thought to be initiated by a viral infection, the effectiveness of type I interferons may be related to their role in viral inhibition. In addition, the autoimmune aspects of MS may be down regulated through a number of different mechanisms since type I IFNs have been shown to inhibit DTH responses (also important in TVID and EAE), alter the homing and trapping of lymphocytes, enhance NK and T-cell activity and cytotoxicity, enhance Fcγ receptors effects (Belardelli

and Gresser, 1996), inhibit T-cell proliferation, and decrease the production of IFN-γ (Noronha et al., 1993).

EAE and Theiler's virus, two animal models for multiple sclerosis, have similar pathologies (Dal Canto et al., 1995). IFN-τ has been shown to prevent the development of the acute form of EAE in NZW mice and to prevent the development of chronic-relapsing EAE in SJL/j mice (Soos et al., 1995). In addition to protecting against both acute and chronic forms of EAE, parental or oral administration of IFN-τ has been shown to alleviate ongoing, chronic, relapsing EAE in SJL/j mice (Soos et al., 1997). The effectiveness of IFN-τ in the treatment of active EAE and its ability to exert an effect when administered orally without the induction of neutralizing antibodies makes IFN-τ a more attractive therapeutic agent for the treatment of MS. Knowing that IFN-τ has proven to be an effective treatment in EAE by preventing acute induction and superantigen reactivation of EAE via suppressor cells (Soos et al., 1995; Majtaba et al., 1997), it was of interest to investigate the effect of IFN-τ on lesion development in the CNS in TVID.

IFN-α and IFN-β are two of the type I interferons that have been investigated as possible treatments for multiple sclerosis. The administration of type I interferons, IFN-α/β, in combination in SJL/J mice infected with the DA strain of Theiler's virus resulted in increased demyelination when given 4.5 months p.i for sixteen weeks (Njenga et al., 2000). IFN-α/β given for the short term resulted in the promotion of remyelination. The authors noted that the mice lost weight during weeks 6-7 of treatment, developed reduced DTH responses to TMEV and decreased numbers of T-cells and B-cells infiltrating the CNS. The treated mice developed antibodies to IFN-α/β which did not appear to affect the efficacy of the treatment. Furthermore the IFN-α/β treatment

did not affect viral replication as measured by *in situ* hybridization quantification of RNA-positive cells. Therefore, the therapeutic effect was not mediated by the antiviral effects of IFN-α/β, but rather by the immunomodulatory functions.

IFN-τ treatment of Theiler's virus-infected mice during late disease results in reduced inflammation and demyelination. When used to treat chronic BeAn TMEV infections, IFN-τ does not significantly change the white blood cell differential of female SJL/J mice when compared to non-treated controls. Occasionally, lymphopenia, neutropenia, and leukopenia are detected in MS patients as a result of IFN-β administration (PRISMS study group, 1998). The data reported in the current study confirms that IFN-τ does not effect circulating WBC counts in contrast to IFN-β.

The data reported here indicates that IFN-τ is an effective therapeutic agent for the treatment of mice with the late stages of TVID, but administration prior to infection and during the early stages of disease can exacerbate clinical presentation. Mice that were given IFN-τ before viral infection were significantly worse clinically and lost more weight than control, infected mice, possibly as a result of the suppression of the Th1 cytokine response during initial infection (Borrow et al., 1993). The H+E staining of the spinal cords of mice treated with interferon-tau before viral infection confirmed the clinical presentation data with an increase in demyelination, status spongiosus, and inflammation. Th1 proinflammatory cytokines including interferon-gamma are required for the immune system to mount an effective antiviral response and decrease viral titers (Rodriguez et al., 1995). It follows then that the administration of an immunomodulator like interferon-tau during early TMEV infection would decrease Th1 cytokine

production and result in an increase in lesion presentation due to increased viral load.

Demyelination in Theiler's virus infection occurs through multiple mechanisms including (a) direct viral lysis of oligodendrocytes, (b) autoimmunity, (c) bystander demyelination mediated by antiviral $CD4^+$ T-cells, and (d) cytotoxic T-cell lysis of infected oligodendrocytes (figure 5.1). It is hypothesized that during early TVID, demyelination is mediated primarily through viral infection, $CD8^+$ cytotoxicity and $CD4^+$ T-cell responses to virus. The autoimmune responses to myelin are only evident at late times during the disease process, between 3 and 9 months p.i. (Miller et al., 1997; Borrow et al., 1998). IFN-τ may be most effective at interfering with the autoimmune aspects of TVID which may explain the reason for it's relative ineffectiveness during the 2-6 months treatment period. The first antiviral DTH response in TVID occurs at 34 days p.i. (Miller et al., 1997). Treatment from 2-3 months may interfere with the viral clearance mechanisms, although further experiments are necessary before definite conclusions can be made.

There was only a slight significant difference in the clinical scores and weights of the mice treated with IFN-τ early in the course of the chronic disease (2-6 month post-infection) compared to the infected controls. All of the 2-6 month infected, treated mice were clinically scored between 2 and 3 before treatment began. It is possible that continued exposure to IFN-τ may be of additional benefit to these mice although further experimentation is required to address this issue.

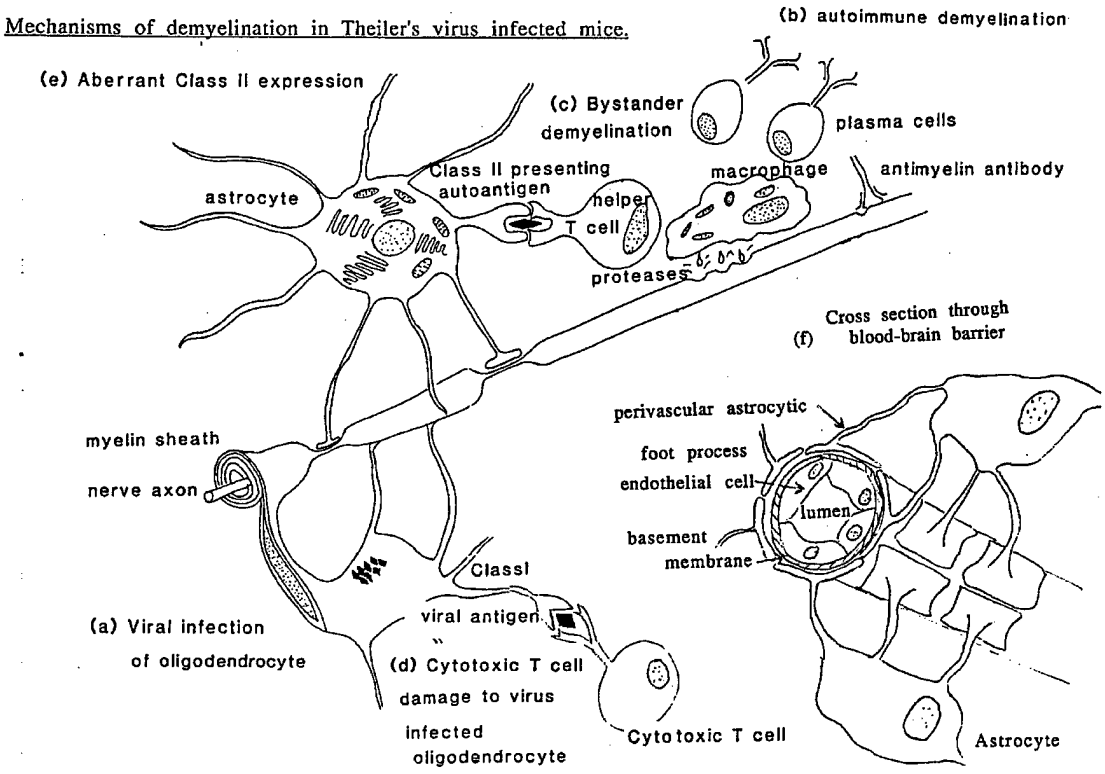

Figure 5.1: Mechanisms of Theiler's virus induced demyelination

During the late stage of demyelinating disease (4-6 month post-infection) IFN-τ treated mice showed marked improvement. At the beginning of treatment, all the clinical scores of the 4-6 month treated mice were between 3 and 4. They gained weight and improved clinically with treatment similar to the other reports of infected mice that were $CD4^+$ T–cell deficient late in disease progression (Welsh et al., 1987; Nicholson et al., 1996). Mice were treated with IFN-τ at four months post-infection in an attempt to mimic the situation in MS where the mean time for diagnosis is two years at which time lesions are well established.

It is interesting to note that the data presented here supports the findings of interferon-tau treatment of EAE mice. As expected, a decrease in inflammation, status spongiosus, and demyelination was observed in the H+E and immunostained slides of the spinal cords of SJL/J mice when interferon-tau was administered from 4-6 months post-infection. This was attributed to the down regulation of proinflammatory Th1 cytokines via interferon-tau administration. However, MOG staining was present in the gray matter of IFN-τ 4-6 month infected treated and non-treated mice that was not present in the healthy controls. IFN-τ administration from 4-6 months p.i. did not improve the incidence of lesions in the lateral funiculi as it did in the ventral and dorsal funiculi.

It is possible that IFN-τ therapy at the late chronic disease stage encourages viral replication via immunosuppression and the change in lesion presentation in the gray matter is due to the imbalance between the virus and immune reactions. It is interesting to note, however, that IFN-τ may have limitations in aiding in disease recovery. One subject

that was clinically scored above 4.0 before treatment did not survive the length of the study. This implies that clinical presentation before treatment may be as important as timing of dosage.

When non-infected mice were dosed with IFN-τ, a marginally significant weight loss was detected three weeks after administration when compared to saline controls. This confirms that the statistically significant weight gain seen in the 4-6 month p.i. treated group was a real effect of treatment. No changes in histology were detected in the non-infected, IFN-τ treated group.

The current hypothesis is that IFN-τ protection in EAE occurs via Th2 cytokine activation as noted by increased IL-10 production (Soos et al., 1997), the inhibition of B-cell and T-cell responses in active and chronic EAE (Majtaba et al, 1998), and the lack of inflammatory lymphocytic lesions in the CNS (Soos et al., 1997) during IFN-τ treatment. TVID susceptible SJL mice given an immunosuppressive cytokine, TGF-b2 showed a reduction in the number of virus infected cells and decreased amount of demyelination (Drescher et al., 2000). The mechanism of action was hypothesized to be TGF-b2 reduced infiltration or activation of virus-infected macrophages into the CNS. Female SJL/J mice infected with the DA strain of Theiler's virus and then given IL-4 or IL-10 or both cytokines in combination, showed marked decreases in demyelination and inflammation (Hill et al., 1998). Thus immunosuppressive cytokines are beneficial in the treatment of TVID. It is important that interferon-tau is given during the chronic demyelinating phase of TVID to suppress the production of autoimmune damage to the CNS by Th1 cytokine expression for the most significant improvement to occur. It is possible that tolerance to interferon-tau may develop if administration continued past 2 months even during the

chronic phase of TMEV. Further investigation into the effectiveness of long-term treatment is necessary.

Due to the fact that ovine IFN-τ is a foreign protein in the murine system, it is expected that antibodies would be produced in the murine model. An antibody response to IFN-τ was detected (figure 4.1) in TMEV infected mice treated with IFN-τ. Similar findings from Soos et al. in the same strain of female mice treated with IFN-τ found that these antibodies were non-neutralizing, therefore, they do not effect the activity of IFN-τ *in vivo* (Soos et al., 1997). IFN-τ given orally did not invoke the production of antibodies (Soos et al., 1997) and therefore it would be interesting to determine if oral administration of IFN-τ to TMEV-infected mice was as effective as i.p. administration.

Reports that IFN-τ can inhibit Th1 cytokine production *in vivo* (Newton et al., 1989, Niwano et al., 1989; Skopets et al., 1992; Assal-Meliani et al., 1993) and upregulate the expression of Th2 cytokines (Chaouat et al., 1995) is further supported by the fact that an increase in IL-10 production is found when IFN-τ is used to treat chronic EAE (Soos et al., 1997) and chronic TMEV infection in SJL/J mice. The increase of IL-10 seen with IFN-τ administration in EAE is also detected to the same degree when IFN-α/β is given orally to treat EAE over a seven week period (Brod et al., 1994) suggesting that IFN-τ functions in a comparative manner to IFN-α/β in the treatment of autoimmune-induced MS like disease.

It is known from previous works that IFN-τ has antiviral (Dereuddre-Bosquet et al., 1996; Johnson et al., 1999; Juste et al., 2000) and immunomodulating properties (Newton et al., 1989, Niwano et al., 1989; Skopets et al., 1992; Assal-Meliani et al., 1993; Tuo et al, 1993; Chaouat et al., 1995). A delayed-type hypersensitivity reaction to

myelin occurs in TMEV-induced demyelinating disease and is proposed to contribute to bystander demyelination during chronic infection (Miller and Gerety, 1990; Miller 1997; Borrow et al., 1998). The data presented here suggest that IFN-τ treatment of chronically infected mice is able to decrease the Th1-induced DTH reaction to myelin that is thought to contribute to the demyelinating process.

IFN-τ has been reported to increase serum corticosterone levels when administered to sheep (Ott et al., 1997). Increased corticosterone could result in immunosuppression, therefore, corticosterone was measured in the experimental mice. IFN-τ administration did not increase corticosterone levels in the mice. Interestingly, high levels of corticosterone were detected in virus infected mice with high clinical scores regardless of treatment group. No statistical correlation was detected between IFN-τ treatment and high corticosterone levels ($p > 0.05$). Based on the preliminary data in this report, it is suspected that the level of circulating corticosterone in chronically TMEV-infected mice may correlate with the level of clinical disease progression (Table 4.2 & Figure 4.4).

Though not much is known about corticosterone and TMEV, previous works show that proinflammatory cytokines can impair normal glucocorticoid function in humans and contribute to neuronal damage in MS patients (Fassbender et al., 1998; Bergh et al., 1999). It stands to reason then that an increase in corticosterone would be seen during the chronic phase of TMEV when Th1 cytokine expression is elevated (Chang et al., 2000). It is curious to note that the highest level of corticosterone reported in this study occurred in the methylprednisolone treated group and the lesions in that group were fairly prominant. It is possible that the dose given was too low to significantly impair the Th1 cytokine damage in the TMEV chronic progressive MS model as seen in MS

patients (Tourtellotte and Haerer, 1965; Millar et al., 1967). A larger sample size is needed to determine if there is a true correlation between serum corticosterone levels and TMEV clinical presentation.

The reports on cytokine mRNA in the CNS tissue of mice infected with TMEV are conflicting due to the fact that different investigators use different assays and perform their experiments during different times of infection. Using the DA strain of TMEV, at 40 days P.I. in SJL/J mice, Sato et al. found that the Th1 cytokines IL-1 and IL-2 were not detectable in the spinal cord whereas Th2 cytokines, IL-10, and Th1 cytokines TNF-α, IL-12, and IFN-γ were elevated when compared to controls. In the brains of the same animals, IL-10, IL-12, TNF-α, IL-1, IL-2, IL-6, and IFN-γ were not detected at 40 days p.i. but, IL-4 production was high in the brain (Sato et al., 1997). It has also been reported that IFN-γ, IL-1, IL-2, IL-6, IL-12, TNF-α, TGF-β1, IL-4, IL-5 and IL-10 cytokine RNA was detected at levels above controls at 60 days p.i. in SJL/J mice also infected with DA (Chang et al., 2000). High levels of lymphotoxin-β, TNF-α, IL-6, and TGF-β were observed in the brains of SJL/J mice infected with DA for 60 days in a separate study (Theil et al., 2000). These findings agree with some of the other work done at 60 days p.i. that does not include spinal cord data. Since there appears to be inconsistencies between 40 and 60 days p.i. with the same TMEV strain, it would be interesting to see if there was a difference in cytokine expression in the CNS of mice infected with the BeAn as compared to the DA strain of TMEV.

The RNA protection assay analysis reported here was performed on spinal cord tissue of mice with the BeAn strain of TMEV at 180 days post-infection. An increase was noted in IL-1, IL-6, IL-10, and IFN-γ in IFN-τ 4-6 month p.i. treated mice as compared to TMEV-infected

controls. The upregulation of IL-10 was expected due to the IL-10 serum ELISA data discussed previously. The increase in IL-1, IL-6, and IFN-γ maybe normal in TMEV BeAn infection but the control, infected mice did not express these cytokines at the same level as the IFN-τ treated mice. Though clinical presentation improved significantly with IFN-τ administration, total recovery was not detected with this treatment possibly due to truncated axons that can not be repaired via remyelination though this was not evident by light microscopy (McGavern et al., 2000). It is also possible that the activation of macrophages through IL-1 and IFN-γ and the production of antibodies through IL-6 expression contributes to the demyelination seen in the IFN-τ 4-6 month treated mice. Further cytokine analysis is required on non-infected mice treated with IFN-τ and larger sample sizes to determine if the upregulation of the Th1 cytokines seen is due to the IFN-τ treatment or TMEV infection.

In conclusion, the increase in serum and spinal cord IL-10, decrease in DTH response to myelin, decrease in inflammation, and decrease in demyelination in the 4-6 month IFN-τ treated group imply that IFN-τ functions as a Th1 immunomodulator when administered during chronic TMEV infection. Elevations in IL-10 would result in decreased DTH responses to myelin and subsequently decreased inflammation and demyelination.

The possibility that IFN-τ may be a therapeutic agent in the fight against MS progression is very exciting, but further work needs to be done with demyelinating animal models to understand the mechanism of IFN-τ. Viral RNA in the CNS needs to be measured as well as *in situ* hybridization cytokine analysis performed to determine if IFN-τ administration halts disease progression via antiviral mechanisms as

well as immunosuppressive means. DTH reactions to virus and myelin epitopes in this model are needed as well to answer the antiviral question. IFN-α/β administration in TMEV-infected SJL/J mice did not decrease viral load so an antiviral mechanism is questionable (Njenga et al., 2000) though IFN-α and IFN-β may function differently when not combined and should be compared to IFN-τ in TMEV *in vivo*.

Challenge of TMEV-infected mice with a secondary viral agent during IFN-τ treatment needs to be looked at since the current research suggests IFN-τ is a Th1 immunosuppressive agent and functions via the same receptor as IFN-α and IFN-β. The possibility that IFN-τ may interfere with the activation of the other type I interferons by receptor competition should also be investigated. Also, oral administration of IFN-τ would be more favorable for human patients as opposed to injections so oral dosage of IFN-τ should be included in future research.

As a side note, it was observed during the removal of the spinal cords of infected mice treated 4-6 months with IFN-τ that the lumbar region was abnormally small and the thoracic area was large in comparison to control TMEV infected mice. The cords of the treated mice appeared to be truncated and contained more dorsal root ganglia originating from the thoracic region than expected. The possibility that IFN-τ may stimulate axonal regeneration or redirection needs to be studied further with the use of axonal neurotracers and dyes.

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APPENDIX

PREPARING VIRUS FOR MOUSE INTRACRANIAL INJECTION

Materials

1. BeAnT$_1$P$_1$ concentration 7.5 x 10^6 plaque forming units

2. Sterile 2mL centrifuge tubes

3. Sterile 1X PBS

4. Metofane®, Schering-Plough Animal Health Corp., Union, NJ, cat# NDC0061-5038-01 or
Isoflurane®, Abbott Labs, North Chicago, IL, cat# 5260-04-03

5. 1mL syringes

6. 27 gauge ½" needles

7. Glass jar with lid

8. Cooler with ice

9. Cotton

10. String

Methods

1. Keep the virus on ice constantly until time of injection.

2. Remove 1mL BeAnT$_1$P$_1$ concentration 7.5 x 10^6pfu/mL from the 80°C freezer.

3. Place 0.5mL of virus into a sterile, 2mL centrifuge tube. Repeat.

4. Centrifuge the tubes for 5 min at 7000RPM to remove cellular debris.

5. Add 1mL sterile PBS to each tube.

6. Take the virus, glass jar with lid and anesthetic to LARR. The virus should be in a cooler with wet ice.

7. Add a small amount of Metofane® to a cotton ball and suspend in the glass jar with lid closed. For Isoflurane®, use 5mL.

8. Place one mouse in the jar.

9. Wait for the mouse to go to sleep in the jar, time varies with Metofane®, 30 seconds for Isoflurane®.

10. Remove the mouse and inject 20uL of the PBS/virus solution intracranially into the right cerebrum.

11. Repeat for each mouse.

12. Place a sign on the animal cage that says, "Anesthetized with Metofane® or Isoflurane® today" and include the date.

Caution!
Only use the Metofane® or Isoflurane® in the fume hood. Always keep the lids on the bottles and the lid on the glass jar when possible. Both chemicals are powerful anesthetics and will affect humans as well.

MOUSE TAIL BLEEDING PROCEDURE

Materials

1. 1 Eppendorf tube/ mouse

2. 1 surgical blade/ 5 mice

3. Heat lamp

4. 1 clean cage

5. Restrainer or 1X 50mL centrifuge tube with breathing and tail holes

6. 50mL 70% ethanol
(100% ETOH, Sigma, St. Louis, MO, cat#E7148 diluted in MilliQ water)

7. Cotton balls

8. Vaseline

9. Rack for Eppendorf tubes

Methods

1. Attach heat lamp to the side of the clean cage.

2. Place one mouse under the heat lamp until the mouse begins wiping it's nose. (approx. 5 min.) The heat will increase the blood flow.

3. Place the mouse in the restraint tube, keeping the tail accessible.

4. Wipe side of tail with ethanol.

5. Make one small nick on the edge of the tail along the vein.

6. Drip the blood into the Eppendorf tube, squeezing the tail if necessary.

7. Collect approx. 0.5mL.

8. Wipe off tail with cotton. Hold until bleeding stops.

9. Apply Vaseline to aid in clotting.

10. Return mouse to cage.

11. Add a petri dish of water to each cage that contains mice that have been bled.

Warnings!
Do not keep the mouse under the heat lamp longer than necessary. Too much heat can kill them!!! Watch the mice for about 5 minutes after all the bleeding is completed. If too much blood is removed, the mice will die within that time frame. Assume total blood volume per mouse equals 6% of the mouse body weight. Do not remove more than 10% of blood volume during bleeding or the mouse will go into shock.

EXTRACTING SERUM FROM BLOOD SAMPLES

Materials

1. Two 0.65mL tubes for each blood sample.

2. One 2mL tube for each blood sample

3. Unplugged, long Pasteur pipettes

4. Pipette bulb

Methods

1. Place blood samples at 4°C for at least 2 hours to cause clot contraction.

2. Transfer all liquid that has not clotted into 0.65mL tubes using a pasture pipette.

3. Cut the lids off of the new 2mL tubes.

4. Place each 0.65mL tube into a topless 2mL tube so they will fit in the centrifuge.

5. Centrifuge for 5 min at 5000 RPM to spin down red blood cells

6. Pipette yellow serum into fresh 0.65mL tubes.

7. Store serum samples at -20°C or -80°C until needed.

Warnings!

Storing the whole blood samples at freezing temperatures will result in the rupture of the red blood cell walls. The serum will be pink and not yellow.

CORTICOSTERONE ANALYSIS OF MOUSE SERUM VIA RADIOIMMUNOASSAY

Materials

1. ImmuChem™ Double Antibody Corticosterone RIA kit, ICN Biomedicals Inc., Costa Mesa, CA, cat#07-120102

2. Gamma counter

3. Test tube rack

4. 10 x 75 mm tubes for RIA

5. Blotting paper

6. Mouse serum, 10μL per mouse

Methods

1. Bring reagents to room temperature.

2. Consecutively number 10 x 75 mm tubes, tubes 1-20 will be used for controls.

3. Dilute mouse serum samples 1:200 with steroid diluent in the kit. Take 10μL of sample and put it in 2.0mL steroid diluent.

4. Add 0.3mL of steroid diluent to tubes 1 and 2.

5. Add 0.1mL steroid diluent to tubes 3 and 4.

6. Add 0.1mL Corticosterone calibrators found in the kit in tubes 5 thru 16 as follows:

tubes 5 and 6 = 0.25ng/mL
tubes 7 and 8 = 50ng/mL
tubes 9 and 10 = 100ng/mL
tubes 11 and 12 = 250ng/mL
tubes 13 and 14 = 500ng/mL
tubes 15 and 16 = 1000ng/mL

7. Add 0.1mL diluted (1:200) controls to tubes 17 thru 20 as follows:
tubes 17 and 18 = control I
tubes 19 and 20 = control II

8. Add 0.1mL diluted mouse serum to tubes beginning with tube 21.

9. Add 0.2mL Corticosterone-^{125}I from kit to all tubes.

10. Add 0.2mL anti-corticosterone from kit to all tubes except tubes 1 and 2.

11. Vortex mix all assay tubes and incubate at room temperature of 2 hours.

12. Add 0.5mL precipitant solution for kit to all tubes.

13. Vortex for approximately 1 minute.

14. Centrifuge all assay tubes at 1000 x g for 15 minutes.

15. Pour off the supernatant.

16. Invert tubes and blot the rim of the tubes on absorbent paper.

17. Count the precipitate in a gamma counter.

ELISA FOR THE DETECTION OF INTERFERON-TAU ANTIBODIES IN MOUSE SERUM

Materials

1. Carbonate coating buffer

2. Serum conjugate

3. 1% BSA in carbonate coating buffer

4. PBS Tween 20

5. ABTS tablets and buffer, Roche, Indianapolis, IN, cat#1204521, cat#1204530

6. Interferon-tau

7. Rabbit anti-mouse IgG horseradish peroxidase conjugated antibody (H+L) diluted in 1X PBS, Sigma, St. Louis, MO, cat#A9044

8. Immulon I flat bottomed 96 well microtiter plate, Costar Corp., Cambridge, MA, cat#3590

9. ELISA plate reader

10. Cloth towel

11. Serum samples

Methods

1. Add 50µl per well of 20µg/mL peptide antigen (interferon-tau diluted in carbonate coating buffer) to columns 1-11 of an Immulon I flat bottomed 96 well microtiter plate. Add 50µl per well of carbonate coating buffer to column 12. Column 1 = negative controls (blanks).

2. Incubate overnight at 4° C (refrigerate)

3. Wash plate 4 times with PBS-Tween 20, then once with distilled-deionized water. Each wash consists of rapidly squirting liquid into each well (with a squirt bottle, this is not quantitative), then aggressively shaking the liquid out of the plate 10 times, and then pounding the plate on a cloth towel 10 times to remove the remaining liquid.

4. Add 100μL per well of 1% BSA (diluted in carbonate coating buffer) to all wells.

5. Incubate overnight at 4° C (refrigerate).

6. Wash plate as above.

Setting up plate

7. Dispense 50μL of serum conjugate to all wells, excluding those wells in columns 2 and 12.

8. Dispense 100μL of a 1:20 dilution of the sample to be tested to the wells in column 2 (sample is diluted in serum conjugate). Positive control is in well 2H 1:20 dilution.

9. Dispense 50μL of the sample to be tested to the wells in column 12.

10. Transfer 50μL from the column 2 wells to column 3. Mix and transfer 50μL from column 3 to column 4. Repeat transfers through column 11, discarding 50μL from column 11 (doubling solutions).

11. Incubate for 40 minutes at 37° C (in a CO_2 incubator).

12. Wash plate as above.

13. Add 50µL/well of Horseradish Peroxidase Conjugated Rabbit Anti-Mouse IgG (H&L) antibody at 1:10,000 dilution (conjugate is diluted in serum conjugate).

14. Incubate for 60 minutes at 37° C.

15. Wash plate as above.

16. Add 50µL per well of substrate, and let develop for 50 minutes at room temperature with ABTS tablets & buffer.

17. Read the absorbence of the wells on an ELISA plate reader at 450nm.

Carbonate Coating Buffer pH 9.6

$NaHCO_3$	0.293g, EM Scientific, Gibbstown, NJ, cat#SX0320-1
Na_2CO_3	0.159g, EM Scientific, Gibbstown, NJ, cat#SX0445-1
D.D. H_20	100mL

Serum Conjugate

NaCl	0.435g, Sigma, St. Louis, MO, cat#S-3014
EDTA	0.018g, Sigma, St. Louis, MO, cat#E-5134
Tris	0.303g, EM Scientific, Gibbstown, NJ, cat#AX0945-3
Tween 20	0.2mL, USB Corp., Cleveland, OH, cat#20605
D.D. H_20	100mL

1% BSA in Carbonate Coating Buffer

BSA	0.2g, Roche, Indianapolis, IN, cat#100 360
Carbonate Coating Buffer	20mL

PBS Tween 20 pH 7.4	9 L solution	
NaCl	72g	Sigma, cat#S-3014
KH_2PO_4	1.8g	EM Scientific, cat#PX1570-1
NaH_2PO_4– 12 H_2O	26.1g	
Or NaH_2PO_4– 7 H_2O	19.535g	EM Scientific, cat#SX0715-1
KCl	1.8g	Sigma, cat#P-5405
Tween 20	4.5mL	USB, cat#20605
D.D. H_2O	9L	

Preparation of ABTS Solution:

ABTS	1 Tablet	Roche, Indianapolis, IN, cat#1204521
Buffer	5mL	Roche, Indianapolis, IN, cat #1204530

Mix shortly before addition to plate, keep bottle wrapped in foil until use

IFN-Tau (from Bazer Lab, Kleberg Bldg.) 1:20 dilution per well

IFN-Tau Sample	6µL	
Serum Conjugate	114µL	

Anti-mouse IgG Peroxidase Conjugate 1:10,000

IgG	0.5µL	Sigma, cat#A9044
Serum Conjugate	4999.5µL	

MEASURING DELAYED TYPE HYPERSENSITIVITY REACTIONS TO CNS MYELIN IN TMEV INFECTED MICE

Materials

1. Metofane®, Schering-Plough Animal Health Corp., Union, NJ, cat# NDC0061-5038-01

2. Glass jar with lid and cotton ball on string

3. Engineer's micrometer, Swiss Precision Instruments, Los Angeles, CA

4. Mouse myelin in PBS

5. 1X PBS

6. 1mL syringes and 26 ½" needles

Methods

1. Anesthetize mouse with Metofane® in the glass jar with the lid on in a fume hood.

2. Measure the ear thickness of each ear with the micrometer.

3. Inject one mouse ear with 20μL of PBS and the other ear with 10μg of mouse myelin in 20μL PBS.

4. 24 hours later, measure both ears again.

5. 48 hours later, measure the ears for the final reading.

NEUROLOGICAL EXAMINATION SCORE SHEET FOR MICE

Mouse_____ Date_____ Rater_____

Posture in cage

Flattened	F
On side	L
Curled	S
Sitting/head low	H
Sitting normal	O
Alert/looks ahead	A
Rearing	R
Vertical jump	J
Circling	C
Pacing	P
Writhing	W

Behavior during removal

Sits quietly	0
Vocal/not resistant	1
Rears/looks ahead	
Runs around	3
Freezes	4
Aggressive/attacks	5

Piloerection (Ruffled Fur)

Y/N

Hair Coat

Clean	0
Slightly soiled	1
Moderate soiled	2
Very soiled	3

Mucus membranes/eye/skin color

Pink	Pale
Dark, cyanotic	Dark, brown
Bright, deep red	

Respiratory Rate

Respiratory Character

Normal	Normal Shallow Deep
Increased Decreased	Labored Rales Gasping
Eye Prominence	Eyelid Closure
Normal	Wide open Closed
Exophthalmos	Slightly drooped
Enophthalmos	Drooping halfway
Seizure Onset	Type of Seiz ure
Spontaneous	Chewing CH
Induced by stimuli	Jacksonian/chewing J
	Jacksonian/initial site I
Length of Seizure	Mild clonic/limbs M
Time in minutes_____	Clonic/body C
	Tonic seizure T
Length of Post-ictal Period	Tonic-clonic seizure TC
Time in minutes_____	Opisthotonus OP
	Emprosthotonus EP
	Spasmodic jumping SJ
	Agonal/asphyxial AS
	Status epilepticus SE
Mental Status	Handling Behavior

Alert	Disoriented	Very easy, totally limp
Depressed	Stupor	Easy, alert, limbs close to body
Coma		Moderately easy, vocal
		Freezes, rigid in hand
		Difficult, squirming, tries to bite

Stereotypic Behavior

Head flick	Head search	
Hallucinatory	Catalepsy	
Compulsive lick	Prancing	
Circling	Waltzing	
Retropulsion	Spatial disorientation	
Rocking	Excessive grooming	

Limb Muscle Tone

Muscle is firm not hard
Muscle is soft, may be atrophied
Muscle is tense and hard

Abdominal Tone

Passive Manipulation

-Place on back and flex each limb

Slight resistance
Completely flaccid
Slightly flaccid
Pain, struggling
Extreme resistance

Moderate resistance
No resistance
Slight resistance
Some resistance, easy rotation
Increased resistance, difficult rotation

Rearing Activity

Did not rear
Falls backward while rearing
Rears on hind limbs with tail
Rears on hind limbs without tail
Falls to side while rearing

Eye Position

Normal	O
Deviated dorsally	D
Deviated ventrally	V
Deviated medially	M
Deviated laterally	L

Head Position

No tilt O Tilt left L Tilt right R

Resting Nystagmus

None O Left L Right R

Static Limb Position

Normal	O
Crossover medially	C
Deviated medially	M
Deviated laterally	L

Positional Nystagmus

Eyes return to center quickly	O
Nystagmus on movement, left	L
Nystagmus on movement, right	R

Tail Elevation

Lifted while walking	0
Occasionally lifted	1
Drags end of tail	2

Pinna Touch Response

No response	0
One twitch	1
More than one twitch	2
Whole body withdraws	3

Startle Response

No reaction	0
Flinch or flicks ear	1
Jumps or violent	2

Visual Orientation

Orients toward object as it enters	O
Orients after well in visual field	S
Orients and responds aggressively	A
Does not orient at all	N

Tail held vertically	3
Tail held 45 degrees	4

Abnormal Gait

None	Knuckling over
Steppage	Crossing over
Spastic	Drags hind limbs
Waddling	Thorax not lifted

Ataxic Gait

None	0
Slight but definite	1
Considerable, no falling	2
Considerable, some fall	3
Considerable,frequent	4
Always falls	5

Locomotor Activity

Reduced activity	0
Resting, slow spatial orient.	1
Vigorous scratching	2
Vigorous movement	3
Sharp, rapid darting	4

Approach Response

Rodent approaches and sniffs	O
No reaction	N
Rodent freezes	F
Pulls away slightly	P
Jumps to avoid object	T
Jumps at object, attacks	J

Paresis

None	0
Weak but ambulatory	1
Non ambulatory	2
Some voluntary movement	3
No voluntary movement	4

Righting Reflex

Rights when turned, either side	O
Rights but impaired, either side	I
Attempts to right but cannot	A
Does not right from right side	R
Does not right from left side	L
Does not right from either side	E

Proprioceptive Positioning

R TL	0	1	2
L TL	0	1	2
R PL	0	1	2
R PL	0	1	2

Extensor Postural Thrust

Prior to contact, extends the PL to bear weight	O
Hindlimbs extended, stumbling on contact	S
On contact hindlimbs not extended	N

Visual Placing		Tail Pinch	
Early vigorous TL extension	0	Walks forward	O
Extension prior to vibrissae contact	1	Sluggishly turns to look	T
After vibrissae contact	2	No response	N
After marked vibrissae contact	3	Freezes with or without vocalization	F
After nose contact	4	Immediately bites	A
Bizarre behavior hampers placing	5	Jumps forward with or without vocals	J
No extension after nose contact	6	Exaggerated response, rapid turning	E

MOUSE PERFUSION

Materials

1. 70% Ethanol (100% Ethanol, Sigma, St. Louis, MO, cat#E7148 diluted in MilliQ water)

2. 10% Formalin, Sigma, St. Louis, MO, cat#HT50-1-2

3. 1X PBS

4. Rompun, Bayer Corp., Shawnee Mission, KS: Sedative and Analgesic, dilute 1:100 in PBS 5uL/10g mouse

5. Ketaset, Fort Dodge Animal Health, Fort Dodge, IA: Anaesthetic 10uL/10g mouse

6. Two way syringe w/ 10mL syringes, 3 way tap, IV tubing, 271/2 gauge needle on end

7. 1cc syringes and 271/2 gauge needles for sedative injection

8. Plastic bags, 1/mouse or 50ml centrifuge tubes.

9. Medical tape for securing mouse

10. Sharp dissecting scissors, forceps, and small scissors for heart

11. Package of paper towels

12. Cage lid

Methods

1. Fill both 10cc syringes, one with PBS, the other with formalin.

2. Fill 1cc syringe with Ketaset and another with diluted Rompun.

3. Inject 100uL of Ketaset and Rompun into mouse i.p.

4. Wait until mouse is immobile and begins "deep breathing" or gasping.

5. Secure mouse on it's back to wire grid with medical tape.

6. Place paper towels under cage to catch blood.

7. Pull on one foot and pinch to check reflex.
If there is no movement, pour ethanol on belly fur.

8. Cut up through the rib cage beginning at the midsection, be careful not to cut the heart with small scissors.

9. Stab the left ventricle with the IV needle, careful not to punch through the heart. Cut the right atrium of the heart.

10. Slowly pump in about 5mL saline to flush out the blood.

11. Slowly switch the 3 way valve to formalin and pump through about 5mL. This will fix the brain tissue.

12. Remove the mouse from the wire rack.

13. Place the mouse right side up and cut between the spinal cord and the brain stem.

14. Place the mouse in a plastic bag or centrifuge tube marked with mouse number.

15. Cover the mouse with 10% Formalin.

16. Refill the syringes. Flush out the IV tubing with PBS to clean out residual formalin.

17. Replace paper towels after each mouse. Keep the towels under the hood until the entire procedure is over.

18. When the dissections are complete, all mouse tissue and paper towels should be placed in biohazard bags and disposed of at LARR.

SAMPLE COLLECTION FOR HISTOLOGICAL ANALYSIS

Materials

1. 70% Ethanol (100% Ethanol, Sigma, St. Louis, MO, cat#E7148 diluted in MilliQ water)

2. 10% Formalin, Sigma, St. Louis, MO, cat#HT50-1-2 or
   4% Paraformaldehyde/4% gluteraldehyde,
   Paraformaldehyde, EM Scientific, Ft. Washington, PA, cat# PX0055
   Gluteraldehyde, EM Scientific, Ft. Washington, PA, cat# GX015305

3. 1X Phosphate buffered saline, PBS

4. Labeled cassette

5. Plastic container (wide opening)

6. Razor blade

7. Surgical instruments

8. Sodium cacodylate-trihydrate, 0.2M, EM Scientific, Ft. Washington, PA, cat#12300

Methods

1. Spleen: if possible, leave in one piece only.

2. Small samples like adrenals and lymph nodes: cut small square piece of lens paper, fold into 4, place inside small funnel, then inside small beaker, wet with PBS, add your sample, fold lens paper so the sample won't get out, place inside labeled cassette, then add 70% ethanol.

3. Spinal cord and brain tissue: if possible try to cut each piece the same size, that way you can have all type of tissue section on each slide. For Brain, remember to keep in formalin and take the same day as perfusion to the histology lab for processing. Leaving the tissue in

70% ethanol overnight can cause artificial spongiform changes. For CNS tissues, it is best to leave the brain in the skull overnight in formalin before removing it for histology. This will prevent artifact in the form of dark staining neurons.

4. Intestine or stomach: flush with sterile PBS before placing sample inside the cassette or it will harden during processing.

DO NOT OVERFIX YOUR TISSUE:
If you need your slides for immunohistochemistry, over fixation will minimize your reaction and decrease antigen retrieval. If the immunohistochemistry is for the spinal cord samples, request formic acid for the decalcification method.

5. If you are collecting tissue for electron microscopy, perfuse with the 4%paraformaldehyde/4% gluteraldehyde instead of formalin. Place the tissue in the same solution in a 50mL tube for 12-16 hours at 4°C. After fixation, dissect out the tissue for EM and place in sodium cacodylate buffer. Process immediately if possible. Store at 4°C.

HISTOLOGY SCORING SHEETS FOR SPINAL CORD SECTIONS

Mouse_____ Rater_____ Date_____

Inflammation
- A. Meninges
- B. White Matter
- C. Grey Matter

Status Spongiosus
- A. Meninges
- B. White Matter
- C. Grey Matter

Demyelination
- A. Meninges
- B. White Matter
- C. Grey Matter

Notes and Comments

Location of Lesion

A. White Matter
1. Dorsal funiculi
2. Lateral funiculi
3. Ventral funiculi

B. Grey Matter
1. Dorsal Horn
2. Intermediate
3. Ventral Horn
4. Medial Grey

Rating System
0.0 No lesion
0.5 Very mild: marginally identifiable lesion
1.0 Mild: definitive lesion, focal to multifocal, limited in severity
2.0 Moderate: definitive lesion, multifocal to regional distribution, extensive severity
3.0 Prominent: definitive lesion, multifocal to diffused distribution, extensive severity
4.0 Very prominent: definitive lesion, multifocal to diffused distribution, very prominently extensive in severity

TRIPURE™ RNA EXTRACTION FROM WHOLE TISSUE

Materials

1. 200mL beaker with wet ice

2. Round bottom Nalgene tubes, two for each tissue sample, Fisher, Pittsburgh, PA, cat#05-562-106

3. TriPure Isolation reagent, 1mL for each 50-100 mg of tissue, Roche, Indianapolis, IN, cat#1667165

4. RNA Zap decontamination spray, Ambion, Austin, TX, cat#9782

5. 95% ETOH, 250mL

6. 75% ETOH, 1mL for each mL of TriPure used

7. DEPC-treated water, 500mL, Ambion, Austin, TX, cat#9915G

8. RNase free tips and microcentrifuge tubes (NEVER USED AUTOCLAVED SUPPLIES – THE CONTAMINATION IS VERY HIGH)

9. 7X soap, ICN, Aurora, OH, cat#76-670-94

10. Kimwipe

11. Nanopure water (Bazer lab has water filter system)

12. Chloroform, 0.2mL per 1mL of TriPure used, Sigma, St. Louis, MO, cat#C2432

13. Parafilm

14. Isopropanol, 0.5mL per 1mL TriPure used, Sigma, St. Louis, MO, cat#I9516

15. Ambion™ Ultrapure water, 100uL per sample, Ambion, Austin, TX, cat#9930

16. Spectrophotometer

17. Power homogenizer

18. 4°C microcentrifuge

CAUTION!

When working with RNA, RNA proteases are constantly in your surroundings. Make sure to spray all pipettes, hands, and surfaces with RNA zap periodically while working. If you touch something that is not sprayed with RNA zap, spray it and your hands again.

Methods

1. Wash the Nalgene tubes in 7X soap. Rinse with distilled water 6X. Squirt RNA zap into each tube and scrub with tube brush. Rinse each tube 6X with Nanopure water. Spray again with RNA zap and dry with Kimwipe.

2. Using the cleaned, round bottom, Nalgene tubes, add 1mL TriPure to every 50-100mg of tissue at room temp. The capacity of the tube should be twice the volume of the tissue plus the TriPure. Record the amount of TriPure used per sample. For samples less than 10mg, use 0.8mL TriPure.

3. Add tissue to the tubes with the TriPure using a sterile pipette tip to remove the tissue from the cryovial.

4. Homogenize the tissue for about 30 seconds. Keep the bottom of the tube in a beaker filled with ice. Make sure to wash the homogenizer with 95% ethanol and DEPC-treated water between each sample. Dry the homogenizer with a Kimwipe.

FOR BRAIN AND SPLEEN SAMPLES: Clarify the homogenate by spinning at 12,000 x g for 10 minutes at 4°C. Transfer the supernatant below the fatty film on top to a fresh round bottom tube.

5. Incubate each sample at room temp for 5 minutes.

6. Add 0.2mL chloroform to each sample for each 1mL TriPure used.

7. Cover with parafilm and vortex each sample for approx. 50 seconds.

8. Incubate at room temp for 15 minutes.

9. Centrifuge at 12,000 x g for 15 minutes at 4°C. The upper colorless phase contains the RNA. The interphase contains the DNA, and the red organic phase is the protein.

10. Transfer the colorless phase into Eppendorf tubes. Each tube will need to hold 0.5mL isopropanol for each 1mL TriPure used. Split into separate tubes if necessary.

11. Add 0.5mL isoproponal for each 1mL TriPure used.

12. Invert the tubes to mix thoroughly.

13. Incubate for 10 min at room temperature.

14. Centrifuge at 12,000 x g for 10 min at 4°C.

15. Discard the supernatant.

16. Add 1mL 75% ethanol to each sample per 1mL TriPure. The RNA pellet can be stored in ETOH for at least a week at 4°C and at least a year at -20°C. Stop here if necessary.

17. Wash the RNA pellet in the ethanol by vortexing.

18. Centrifuge the sample at 7500 x g for 5 min at 4°C. Pour off the supernatant.

19. Remove the excess ETOH by inverting on the bench top and wicking the extra ETOH off the sides with a Kimwipe. Be careful not to totally dry the pellet or touch it with a Kimwipe.

20. Resuspend the pellet by adding 30uL per tube of Ambion Ultrapure water. Combine the like tubes and keep the samples on ice after resuspending. Record final volume of samples.

21. Make a 1:60 dilution of each sample. 2uL sample into 118uL DEPC-treated water.

22. Place stock RNA into -80°C.

23. Take diluted samples to Bazer lab and read on the spectrophotometer nucleic acid program setting. Load 100uL of each dilution into the cuvette. Be careful to rinse the cuvette with DEPC-treated water in between each reading.

24. Record the 260nm absorbence and the 260nm/280nm ratio. RNA samples should have a 260nm/280nm reading between 1.6 and 2.0.

To calculate total RNA:
260nm reading x dilution factor x 40 / 1000 = ug/uL

Dilution factor = 60 RNA constant = 40

EXTRACTING RNA FROM DNA CONTAMINATED SAMPLES

Materials

1. Rq1 RNase free DNase, Sigma, St. Louis, MO, cat#D7291

2. DEPC water, Ambion, Austin, TX, cat#9915G

3. Phenol:chloroform:isoamyl alcohol 25:24:1, Sigma, St. Louis, MO, cat#P3803

4. RNase free 200uL tips and 2 mL tubes, Ambion, Austin, TX, cat#12650, cat#12425

5. Chloroform, Sigma, St. Louis, MO, cat#C2432

6. 3M Sodium acetate, Sigma, St. Louis, MO, cat#S2889

7. 100% Ethanol, Sigma, St. Louis, MO, cat#E7148

8. 70% Ethanol (100% Ethanol diluted in MilliQ water)

9. Ambion Ultra-pure water, Ambion, Austin, TX, cat#9930

Methods

Day 1

1. Spin samples quickly to bring all liquid to the bottom of the tube.

2. Add 1uL Rq1 RNase free DNase to each 14uL of sample.

3. Incubate at 37°C for 15 minutes in a heat block or water bath.

4. Add 185uL DEPC water per 1uL Rq1 to each sample.

5. Add equal volume phenol:choloroform:isoamyl alcohol (200uL for 1uL Rq1).

6. Vortex 20 seconds and centrifuge at full speed for 2 minutes.

7. Remove the top layer in the tube and place in a separate tube.

8. Add equal volume of chloroform to the new tube.

9. Vortex 20 seconds and centrifuge at full speed for 2 minutes.

10. Remove the top layer in the tube and place in a separate tube.

11. Add 1:10 volume 3M sodium acetate pH 7.4 (20uL into 200uL)

12. Add 2.5X 100% ethanol and store at -20°C for 4-24 hours.

Day 2

13. Spin at 4°C at 20,000 x g for 30 minutes.

14. Carefully pour off ethanol.

15. Add 1mL 70% ethanol to each sample and spin at 4°C at 20,000 x g for 15 minutes.

16. Pour off ethanol and invert tubes on bench to dry.

17. Use a Kimwipe to absorb excess ethanol but do not touch the pellet.

18. Reconstitute in 35uL Ambion Ultra-pure water and keep the samples on ice.

19. Make a 1:60 dilution (2uL/118uL) of sample into DEPC water.

20. Place undiluted samples into the -80°C.

21. Read diluted samples on the spectrophotometer nucleic acid setting.

IMMUNOSTAINING FOR CNS MYELIN

Materials

1. 1 Coplin jar per 10 slides

2. 10mM Sodium citrate pH 6.0 (Sigma, St. Louis, MO, cat#S4641 diluted in MilliQ water)

3. Xylene, Sigma, St. Louis, MO, cat#X2377)

4. 100% Ethanol, Sigma, St. Louis, MO, cat#E7148

5. 95% Ethanol (100% Ethanol diluted in MilliQ water)

6. 70% Ethanol (100% Ethanol diluted in MilliQ water)

7. 30% Hydrogen peroxide, EM Scientific, Gibbstown, NJ, cat#HX0635-2

8. Methanol, Sigma, St. Louis, MO, cat#17-5

9. 1X PBS

10. PBS/BSA 2%

11. PBS/BSA 2% with 1% triton

12. Myelin/oligodendrocyte Ab-1 (Clone CE1) NeoMarkers, Inc., Fremont, CA, cat#MS-245-P

13. Rabbit anti-mouse IgG horseradish peroxidase conjugated, Sigma, St. Louis, MO, cat#A9044

14. 0.05M Tris-HCl pH 6.8 (Sigma, St. Louis, MO, cat#T2413 diluted in MilliQ water)

15. 3,3'Diaminobenzidine tetrahydrochloride (DAB) tablets, ICN, Aurora, OH, cat#980681

16. Normal rabbit serum for blocking, Sigma, St. Louis, MO, cat#R-9133

17. Mouse IgM antibody, Sigma, St. Louis, MO, cat#M1520

18. Mayer's hematoxylin solution, Sigma, St. Louis, MO, cat#MHS-16

19. 250mL glass cuvette

20. Aluminum foil

21. Kimwipe

22. Pap pen, The Binding Site Inc, San Diego, CA, cat#AD100.5

23. Humidified chamber made of plastic container with wet paper towels inside

24. Glass 50mL beaker for DAB solution

25. MilliQ water

26. DAB waste bottle and dry waste bag

27. Q-tips

28. Glycerol gelatin for slide mounting, Sigma, St. Louis, MO, cat#GG-1

29. 3mL syringe

30. 40mm-50mm cover slips, 1 per slide

31. Parafilm

Methods

Day 1

1. Turn on 80°C water bath with Coplin jar containing 50mL 10mM Sodium Citrate 1 hour before deparaffinizing slides

2. Deparaffinize slides through xylene and graded alcohol

Xylene ..... 5 min

Xylene ..... 5 min

100% Ethanol..... 3 min
100% Ethanol..... 3 min
95% Ethanol..... 3 min
70% Ethanol..... 3 min
Mix Quenching Solution

3. Quench sections for 30 minutes in 0.3% H_2O_2 in methanol.
* Light sensitive, cover with foil.
(0.5mL 30% H_2O_2 in 49.5mL methanol in Coplin jar)
(2.0mL 30% H_2O_2 in 198mL methanol in glass cuvette)

4. Antigen retrieval
30 min @ 80°C in 10mM (0.01M) Sodium Citrate, pH 6.0 in a separate Coplin jar (six slides per jar).

5. Rinse slowly 2X in dH_2O in Coplin jar after cooled to Room Temperature (45 min). Fill Coplin jar with distilled water.
Wick off dH_2O around tissue with Kimwipe and draw circle with pap pen.

6. Rinse 3X with PBS and then rinse fourth time and leave the sections in PBS for 5 minutes.

7. Rinse with PBS/BSA(2%) as per step 5.

8. Rinse again with PBS/BSA(2%), dry around sections and put on 160µL 10% Normal Rabbit Serum, RT, 20 minutes in humidified chamber w/ lid on top.(120 NRS from -80°C + 1080PBS/BSA(2%), good for 6 slides).

9. Prepare primary Ab and IgM control. Ab and IgM diluted in PBS/BSA(2%).

Stock oligodendrocyte ab = 200µg/mL IgM
For 6 slides = 1036.8µL PBS/BSA + 43.2µL primary antibody
(Vortex and then centrifuge @ 12,800g for 3 minutes)

IgM (made in mouse) stock = 1000 µg/mL
(Vortex and then centrifuge @ 12,800g for 3 minutes)

10. Remove Rabbit serum and dry around sections. Add 160µL ab, PBS, or IgM to slides. INCUBATE slides for 14-15 hrs, 4°C (one section with Ab, the other with IgG, and the third section with PBS/BSA) in humidified chamber.

DAY 2

11. Remove DAB bottle from the -20°C freezer

12. Rinse slides with PBS/BSA(2%) containing 1% triton. (as per step 5, cuts down on background)

13. Rinse with PBS (as per step 5); 2X

14. Rinse with PBS/BSA(2%) containing 1% triton. (as per step 5)

15. Rinse with PBS/BSA(2%) containing 1% triton, dry around sections.

16. Prepare 2°AB (HP conjugated rabbit anti-mouse)
diluted in 5% NRS and PBS/BSA(2%)
(1:200 dilution stock at 1:10 dilution) located in -20°C
(60µL antibody + 1140µL PBS/BSA(2%)/NRS for 6 slides)
Vortex and spin similar to 1° antibody

17. Dry around sections and add 160µL 2° antibody to all slides.
INCUBATE for 1 hour at room temp in humidified chamber w/ lid on top.

18. Add DAB tablet to 8.33mL Tris-HCl in DAB beaker –
WRAP BEAKER IN FOIL.

19. Rinse with PBS/BSA(2%) containing 1% triton, (as per step 5).

20. Rinse with PBS (as per step 5). 2X

21. Rinse with PBS, then with Tris-HCl (0.05M concentration).

22. Dry around sections and apply 160uL peroxidase solution per slide (Add H_2O_2 just before use; in beaker with foil, dab tablet and Tris-HCl), wait till color develops (10 min) grind tablet with pipette tip before use
PEROXIDASE SOL'N = 5mg DAB + 8.33mL Tris – HCl(0.05M) + 83.33µL H_2O_2(3%) DAB: 3,3- diaminobenzidine-HCl.

PLACE DAB WASTE AND TRIPLE RINSES OF DAB BEAKER IN WASTE BOTTLE IN THE FUME HOOD.

23. Rinse in d H_2O, 2 changes, 5 minutes. Place all rinses in DAB waste bottle.

24. Counter stain with filtered Hematoxylin (10 seconds).

25. Rinse with ddH_2O, 4 changes, 3 minutes.

26. Rinse with PBS, then ddH_2O.

27. Let slides air dry in fume hood.

28. Place DAB gloves and pipette tips in DAB dry waste in fume hood.

29. Cover all solutions with parafilm and store at 4°C.

30. Remove pap pen from dry sections with xylene in fume hood using a Q-tip. Be careful not to touch the tissue with the xylene.

31. All approx. 0.5mL per slide of mounting media on the bottom edge of the slide in a line using 3mL syringe w/o needle. Also add mounting media to the underside of a cover slip (40mm-50 mm) in the same manner.

32. Slowly lower cover slip onto the slide starting at the bottom and slowly lowering slide. Mounting media will wick up the slide from the bottom. Avoid bubbles. Push out and bubble by gently rolling a Q-tip across the cover slip.

33. Let the slides dry for 1 hour before viewing.