THE EFFECT OF STRESS ON THE NEUROPATHOGENESIS OF THEILER'S VIRUS INDUCED DEMYELINATION AS AN ANIMAL MODEL OF MULTIPLE SCLEROSIS

A Dissertation

by

WENTAO MI

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 2005

Major Subject: Genetics

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ABSTRACT

The Effect of Stress on the Neuropathogenesis of Theiler's Virus Induced Demyelination as an Animal Model of Multiple Sclerosis. (August 2005)

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Stressful life events have been associated with the onset and/or exacerbation of multiple sclerosis (MS). To investigate the effects of stress on the pathogenesis of MS, we employed restraint stress (RST) in the Theiler's virus-induced demyelination (TVID) model, an animal model for human MS. Intracerebral inoculation of susceptible strain of mice with Theiler's murine encephalomyelitis virus (TMEV) results in a biphasic disease – an acute encephalomyelitis and chronic demyelination. The establishment of persistent viral infection is critical in inducing immune-mediated demyelination during the chronic disease. The exposure of mice to RST prior to viral infection produced a stress response as evidenced by elevated circulating corticosterone (CORT). To further study the effect of stress on the immune response to TMEV infection and demyelination, we first examined the cytokine and chemokine response during the acute TMEV infection. We demonstrated that RST down-regulated the virus-induced expression of chemokines, Ltn, IP-10, RANTES, and pro-inflammatory cytokines, TNF-α, IFN-γ and LT-β in both the brain and spleen during early infection. Histologically, a decreased pattern of inflammation was observed in the brain of restrained mice as compared to non-restrained mice. The increased viral titer was

noted in the CNS of restrained mice and was correlated with the decreased production of pro-inflammatory cytokine, suggesting an impaired immune response by RST. Secondly, the duration of stress on the late demyelination was investigated. Repeated and chronically stressed SJL/J mice developed an early onset of clinical signs and a delayed onset was observed in acutely stressed mice. Both acute and chronic RST suppressed the antibody response to TMEV and stressed displayed a higher incidence of demyelination than non-restrained mice. Axonal loss was also noted in chronic stressed mice. Additionally, RST caused an increased systemic viral infection in extraneural organs during the acute infection and cardiotropic TMEV was isolated from the heart of stressed mice. Taken together, stress resulted in profound immunsuppression during acute infection, which may consequently increase the incidence of demyelination. The present study may be generalized in human MS which is potentially triggered by viral infection.

DEDICATION

This dissertation is dedicated to my beloved family and friends.

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I INTRODUCTION

1. Multiple sclerosis

Multiple sclerosis (MS) is the most common autoimmune diseases involving the central nervous system (CNS) and it affects approximately 250,000 to 350,000 individuals of the US population. MS afflicts females twice as often as males. The MS patients usually experience chronic, progressive, intermittent episodes of neurological dysfunction resulting from demyelination and/or axonal damage. The most common symptoms may involve deficits of sensation, motor, autonomic and neurocognitive function. Based on clinical manifestation, the course of MS can be classified as relapsing-remitting MS (RRMS) or primary progressive MS (PPMS). RRMS accounts for the majority of MS patients (85-90%), and is characterized by recurrent attacks of neurological dysfunction, persisting for days or weeks and then gradually waning. For some RRMS patients, the extent of recovery from each flare-up decreases with time, thus leading to a worse disability with each attack. 10-15% MS patients may present as PPMS, which initiates as a subtle onset but with steady progress and a gradual clinical decline (Hafler, 2004; Sospedra and Martin, 2005).

The etiology of MS remains unclear, but it may result from a complex interplay of environmental and genetic factors. The most persuasive genetic evidence comes from twin studies showing a higher incidence of MS in monozygotic twins than dizygotic pairs. Also, first-degree relatives of MS have an increased risk of MS affliction. Human

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This dissertation follows the style of Journal of Neuroimmunology.

Leucocytes Antigen (HLA)-DR and DQ genes have been associated with MS, suggesting an immunological related pathogenesis of MS (Sospedra and Martin, 2005). In addition, epidemiological studies showing the discordance of MS in monozygotic twins indicate an acquired nature of the disease. Infectious agents, such as virus and Chlamydia, have been implicated in MS, as evidenced by the consistent finding of oligoclonal bands, high concentrations of IgG in MS patients with activity against several viruses. However, no virus has ever been consistently isolated from the brain of MS patients; and viral and bacterial infections are often thought to be environmental inciters of MS (Hartung, 2005). Therefore, MS affects genetically predisposed individuals and is triggered by environmental factor such as infection. Gender, stressful life and geographic gradients also played a role in the disease.

The pathology of MS includes demyelination, variable degrees of axonal damage (axonal loss and transection), perivascular inflammatory infiltration and astrogliosis (plaque). The multiple sharply demarcated plaques in the white matter of the CNS (brain or spinal cord) are the hallmark of MS. The inflammatory infiltrates consist of oligoclonal T cells, monocytes with occasional B cells and infrequent plasma cells. Macrophages are frequently found in the center of active plaques and contain myelin debris, accompanied by decreased numbers of oligodendrocytes. Whereas inflammation is less prominent and is mainly located at the rim of the plaque in the chronic-active lesions (Halfer et al., 2004). Lucchinetti et al. (2000) described four different patterns of lesions in acute demyelinating pathology, suggesting more than one mechanism contributes to pathology. All patterns displayed prominent inflammation with T cells and macrophages. Pattern I and II both showed perivenular distribution of demyelinated

areas with sharply demarcated edges, accompanied by remyelination. Variable oligodendrocyte losses were seen, but apoptosis was rare. The major feature distinguishing pattern I and II was the prominent deposition of immunoglobulin and complement C9neo throughout the lesion. In pattern III, preservation of a rim of myelin was frequently seen around the inflamed vessels, and the plaque border was ill defined. Concentric alternating rings of demyelinated and myelinated tissue were found at the periphery of the lesion. Within the lesion, there was pronounced loss of oliogodendrocytes with apoptosis, and preferential loss of myelin-associated glycoprotein (MAG) relative to other myelin proteins, such as myelin basic protein (MBP) and proteolipid protein (PLP). In pattern IV, demyelination was associated with the death of oligodendrocytes at the plaque edge, but no morphological features of apoptosis. No remyelination or Ig deposition was observed in pattern II and IV.

The immunological mechanism involved in the pathogenesis of MS primarily involves $CD4^+$ autoreactive T cell-mediated inflammation of the CNS and subsequent damage to myelin. Other immunological components, such as $CD8^+$ T cells, antibodies, complement also contribute to the pathogenesis of MS (Sospedra and Martin, 2005). Therapies for MS are derived from understanding the immunopathophysiology of MS, including immunosuppressive drugs, such as cyclosphosphamide (Cytoxan), cladribine (2-CdA, Leustatin), mitoxantrone, interferon-β, corticosteroids; MHC-binding protein that engages T cell receptor, glatiramer acetate (GA) etc (Hafler, 2004).

2. Theiler's virus induced demyelination (TVID) as a viral model for MS

2.1 Theiler's virus and TVID

Theiler's murine encephalomyelitis virus (TMEV or Theiler's virus) was first isolated by Max Theiler in 1930, from the CNS of mice with spontaneous flaccid paralysis of the hind leg (Theiler, 1934; Theiler and Gard, 1940). Theiler's virus is a natural enteric pathogen of mice with a rare incidence of spontaneous CNS infection. It is an RNA virus and a member of the Cardiovirus genus in the Picornaviridae family. The genome of Theiler's virus consists of single-stranded RNA of positive polarity comprising approximately 8,100 nucleotides. It codes for 12 proteins arranged in the order 5'-L, VP4, VP2, VP3, VP1, 2A, 2B, 2C, 3A, 3B, 3C, 3D-3'. The 76-amino-acid long L protein is a zinc-binding metalloprotein (Chen et al., 1995). The expression of L protein has been shown to interfere with immediate-early interferon production (Delhaye et al., 2004; van Pesch et al., 2001); and the mutation of L protein is associated with attenuated neurovirulence in mice (Calenoff et al., 1995), suggesting its critical role in the virus-host interaction. VP4, VP2, VP3, and VP1 are capsid proteins. Proteins 2A, 2B, 2C, 3A, 3B, 3C, and 3D are required, either directly or indirectly, for viral RNA replication (reviewed by Oleszak et al., 2004). Theiler's virus is divided into two subgroups based on differing biological characteristics: (i) the highly virulent strains, such as GDVII and FA, produce a fatal encephalitis in all strains of mice; and (ii) the less virulent, known as Theiler's originals (TO), such as BeAn and DA, cause a chronic, immune mediated, inflammatory demyelinating disease in susceptible strains of mice, such as SJL/J and CBA mice, known as Theiler's virus-induced demyelination (TVID), which resembles human MS in (i) white matter demyelination of CNS with inflammation; (ii) recurrent

demyelination in certain strains of mice; and (iii) demyelination related spastic paralysis (Lipton et al., 1986).

Experimentally, TVID is induced by intracerebral inoculation of less virulent Theiler's virus into susceptible strains of mice, such as SJL/J and CBA, and is characterized by a biphasic disease. During the early phase TVID, TMEV preferentially infects neurons within gray matter, causing acute encephalomyelitis which lasts about 1-2 weeks. In the later demyelinating phase, TMEV predominantly persists in CNS macrophages/microglia and to a lesser extent in oligodendrocytes and astrocytes, and eventually leads to demyelination, which usually occurred at 3-4 week post infection. The model for MS derived from the chronic disease, known as Theiler's virus-induced demyelination, has been widely used in the study of MS (Lipton et al., 1986; Oleszak et al., 2004).

The susceptibility or resistance to TVID is genetically determined by both major histocompatibility complex (MHC) and non-MHC genes. Using morphological criteria in congenic strains of mice to define the resistance or susceptibility, mice with f, p, q, r, s, or v haplotypes on a C57BL/10 background are susceptible to TVID. SJL/J, DBA/1, DBA/2, SWR, PL/J, and NZW strains of mice are susceptible to TVID. Whereas b, d, or k haplotypes on the same background are resistant and BALB/c, C57BL/6, C57BL/10, C57/L, and 129/J are resistant to TVID (Lipton and Melvold, 1984; Rodriguez and David, 1985).

Host responses to TMEV during early infection are also critical in determining either successful viral clearance or persistence of virus in the CNS with the development of demyelination, which requires (i) effective immune response in clearing the virus from

the neurons to prevent fetal encephalitis; (ii) incomplete elimination and persistence of virus in the CNS; (iii) induction of immune response to myelin damage due to viral persistence (Drescher et al., 1997).

2.2. Immunopatholgy of TVID

2.2.1. A timely and effective cytotoxic T cells (CTL) mounted by a resistant strain of mice

The resistance to TVID is closely related with cytotoxic T cells (CTL), as evidenced by a passive transfer of CD8+ T cells from resistant mice to susceptible mice, which protected the latter against demyelinating disease (Nicholson et al., 1996). The functional CTL is highly dependent on the recognition of class I MHC complex on antigen presenting cells (APC) by CD8+ lymphocyte. The K and D loci of mouse MHC encode proteins which comprise the classical class I molecules involved in antigen presentation, and the susceptibility of TMEV infection and demyelination has been mapped to the D loci. Lipton et al. (1995a) showed that mutation in H-2D^b loci resulted in decreased resistance, and inactivating H-2D^b gene of resistant C57BL/6 mice altered the resistance and made them susceptible to infection. The introduction of the H-2D^b gene of C57BL/6 mice into the genome of susceptible H-2^q FVB mice also confers the latter resistance and decreased inflammatory lesions (Azoulay et al., 1994; Mendez-Fernandez et al., 2003). Moreover, recognition of predominant H-2D^b–restricted viral peptides, $VP2_{121-130}$, $VP_{165-173}$, and $VP_{110-120}$ has been identified in BeAn or DA infected C57BL/6 (H-2D^b) mice, which are resistant to viral persistence and demyelination (Lyman et al., 2002). In contrast, a lower CTL response was detected in susceptible mice, suggesting an ineffective CTL-mediated viral clearance in these mice (Dethlefs et al., 1997). Although

both H-2K and H-2D were expressed in the CNS, and both K- and D-restricted virus-specific CTL were generated in the spleen of susceptible and resistant mice, only the D restricted CTL response was detected in the CNS following TMEV infection (Lin et al., 1997). Therefore, H-2D region is closely related with early viral clearance in TMEV infection.

Experiments with knockout mice further indicated the importance of CTLs in resistance to TVID. β2-microglobulin (β-2m)-deficient mice on an H-2^b background, derived from a C57BL/6 and 129/Ola cross failed to eliminate the virus and developed demyelination (Fiette et al., 1993). β2m-deficient SJL mice displayed similar disease incidence rates to wild-type controls; however, the mice demonstrated earlier onset of clinical disease, significant elevation in persisting viral titers, elevated in vitro responses to TMEV and myelin proteolipid (PLP) epitopes, and significantly higher levels of CNS demyelination (Begolka et al., 2001). Disruption of β2m gene in resistant (C57BL/6F 129/J) (H-2b) mice rendered susceptibility to demyelination, but also resulted in extensive remyelination of oligodendrocytes and Schwann cells as compared to wild type (Miller et al., 1995). These results suggest a potential role of β-2m in the susceptibility to TMEV due to a decrease in class I MHC molecule expression and CTLs, and the development of demyelination and remyelination, which are primarily mediated by $CD4^+$ T cells, but the role of $CD8^+$ T cells was noted as well.

Perforin is a glycoprotein whose expression is mainly confined to $CD8^+$ T cells and NK cells. Upon cell-cell contact, perforin is released onto the target cell, causing permeabilization of membrane, which leads to the death of the cell. Disruption of perforin gene in C57BL/6 mice results in high mortality of mice which died of

encephalitis due to high viral titers, suggesting that the early disease is perforin dependent (Rossi et al., 1998). Taken together, an early and rapid CTL, versus a delayed and insufficient CTL response, account for, in part, the differing susceptibility/resistance to TVID in the different stains of mice.

2.2.2. Both CD4+ and CD8+ T cells contribute to the demyelination in susceptible strain of mice

Flow cytometry and histopathology revealed that CD4+T cells are one of the major cell types infiltrating into the CNS in TVID. MHC II-restricted $CD4^+$ T cells that recognize both viral peptides and myelin peptides were identified, and were associated with DTH, indicating the role of $CD4^+$ in myelin damage by epitope spreading (Borrow et al., 1998; Miller et al., 1997; Welsh et al., 1987 and 1989). Deletion of CD4 gene ($CD4^{-/-}$) in B6 mice results in viral persistence and demyelinating disease (Murray et al., 1998), implying that $CD4^+$ T cells are one of the principle factors contributing to the resistance and that the impaired viral clearance may be due to decreased neutralizing antibody secretion of B cells, because class II MHC is required for activation of B cells by $CD4^+$ T cell. Class II MHC-deficient mice ($RHA\beta^{-/-}$) exhibit susceptibility to persistent infection and demyelination, with diminished lesion size as compared to SJL mice (Fiette et al., 1996), which is possibly explained by reduced $CD4^+$ mediated DTH response as a result of the absence of MHC II.

The role of $CD8^+$ T cells in demyelination is controversial: depletion of $CD8^+$ T lymphocytes resulted in reduced myelin damage in the CNS of TMEV-infected mice (Rodriguez and Sriram, 1988). Demyelination was detected in resistant B6 mice

deficient for either the $CD4^+$ or the $CD8^+$ T cells, accompanied by deficient viral clearance; however, $CD4^+$-deficient mice developed delayed DTH to viral antigen, increased demyelination and severe neurological deficit, whereas these changes were minimal in $CD8^+$-deficient mice (Murray et al., 1998). Thus, the discrepancy in the role of $CD4^+$ and $CD8^+$ T cells reflects differential underlying mechanisms, of which the elimination of virus is mainly $CD8^+$-dependent, and the demyelination is primarily $CD4^+$-determined, but both arms of the immune response contribute to the pathogenesis of TVID.

2.2.3. NK cell protects against acute encephalitis

The NK cell activity is mediated by nonclassical MHC I molecule, CD1. A higher level of NK cell activity was found in resistant C57BL/10 mice than susceptible SJL/J mice, which was negatively correlated with viral titers in the CNS. However, depletion of NK cells using NK 1.1 resulted in increased inflammation at early disease, but no changes in demyelination (Paya et al., 1989). Therefore, as innate immunity, NK cell plays a major role in protecting against gray matter disease and a minimal role in demyelination.

2.2.4. Macrophage/microglia habors virus and mediates myelin damage in susceptible mice

Macrophage/microglia serves as a reservoir for TMEV with limited replication, and is responsible for viral persistence in the CNS of susceptible mice (Clatch et al., 1990). The virus can be located in the cytoplasm, but not in phagolysosomes, suggesting that

virus has effectively infected the macrophage, rather than being phagocytocized by macrophages (Lipton et al., 1995b). Activation, as evidenced by TNF-α production, is required for macrophage/microglia infection (Shaw-Jackson and Michiels, 1997). Microglia, as the resident CNS macrophages, can be activated into professional antigen presenting cells (APCs) following TMEV infection and present viral or myelin epitopes to $CD4^+$ T cells, thus leading to demyelination (Olson et al., 2001). During the chronic phase, foamy macrophage can be seen in the demyelinating region of susceptible mice, indicating a phagocytosis of myelin by macrophage/microglia (Oleszak et al., 1997).

2.2.5. Cytokines, adhesion molecules and other soluble factors serve as mediators in the viral clearance and demyelination

A broad spectrum of cytokines has been implicated in the early viral clearance and late demyelination in TVID. For example, IFN-γ may play a critical role in protection from virus infection. Genetic mapping studies have identified the *ifng* locus on chromosome 10, a non-H-2 region. Using depletion or neutralizing antibody to IFN-γ, or IFN-γ receptor knockout mice, these studies demonstrate IFN-γ is required for viral clearance (Fiette et al., 1995). TNF-α, a strong inducer of inflammatory cytokines, appears to be responsible for direct destruction of myelin and oligodendrocytes (Selmaj and Raine, 1988). Soluble mediators, such as nitric oxide (NO), are produced by activated microglia in both acute and chronic phase. Recent studies have shown the toxicity of NO to oligodendrocytes (Baud et al., 2004) and axons (Garthwaite et al., 2002). Unlike the function of other immune cells, such as CTL, where deletion or transfer can dramatically alter the resistance or susceptibility, these soluble mediators

play a regulatory role, and especially, cytokines may function as a network in the pathogenesis of TVID.

2.2.6. Humoral responses in susceptible strains of mice contribute to ameliorating the onset of demyelination

The involvement of humoral response in TVID is evidenced by the detection of antiviral antibodies in the CNS of TMEV-infected SJL mice and the presence of B cells in the demyelinating lesions as revealed by histopathology. Antibodies were found at a higher level in the CNS than in the serum, suggesting a local response. Neutralizing antibody is predominantly targeted to the VP1 and VP2 viron capsid. The epitope of the antibody differs in resistant and susceptible strains of mice, with efficiency in neutralizing virus and thus helping to eliminate TMEV from the CNS in resistant mice (Oleszak et al., 2004). It has also been demonstrated that passively transferred anti-TMEV antibody could successfully protect $CD4^+$ T cell-deleted CBA mice from lethal during the acute disease (Borrow et al., 1993). These results indicate the role of humoral response in the viral clearance of resistant mice. However, the antibody response alone is not sufficient in clearing virus, which is predominantly mediated by cellular immunity, such as CTL.

Depletion of B cells by goat anti-μ (anti-IgG), or depletion of complement by treatment with cobra venom factor, leads to more severe demyelinating lesions in SJL mice. Resistant strains of mice (C57BL/10, C57BL/6, and B10.D2) did not develop demyelinating disease after treatment with goat anti-μ. These findings suggest that humoral responses in late chronic demyelinating stage in susceptible mice contribute

more to ameliorating or delaying the onset of demyelination than to clearing the virus (Oleszak et al., 2004).

2.3. $CD4^+$ T cell mediated demyelination by epitope spreading

TVID is an autoimmune CNS disease mediated by virus-specific and/or myelin-specific $CD4^+$ T cells-induced delayed-type hypersensitivity (DTH) to myelin components via epitope spreading (Borrow et al., 1998 Miller et al., 1997; Welsh et al., 1987 and 1989). Epitope spreading is an acquired T cell autoreactivity generated in a manner of *de novo* priming of T cell to damaged tissue caused by other stimuli, such as viral infection. In TVID, virus-specific Th_1 cells recognize virus epitope presented as a result of persistent CNS infection and initiate self tissue destruction, including myelin, through an inflammatory response known as the bystander effect. Damaged myelin components are released as novel antigen is presented to T cells, resulting in the priming of myelin-specific $CD4^+$ T cells, which mediate demyelination. The evidence of epitope spreading was extensively documented by Miller and colleagues. Virus-specific CD4+ T cells are observed as early as 1 week post infection and chronically persist in the CNS. While myelin-specific T cell responses first appear approximately 50-60 days post-infection and persist throughout the chronic disease. Further kinetic and functional studies rule out the theory that T-cell responses to the immunodominant myelin proteolipid protein epitope (PLP139-151) result from cross-reactivity between TMEV and self epitopes (Miller et al., 1997). The T cell response also displayed an ordered progression throughout the disease process, in which a sequential DTH by virus-specific T cell to immunodominant $VP2_{70–86}$, $VP3_{24–37}$, then myelin-specific $PLP_{139–151}$ and other

myelin components is documented (Katz-Levy et al., 2000). The clinical symptoms first appeared at 30-40 day post-infection, a time frame between the first detection of virus-specific and myelin-specific T cells, indicating demyelination in TMEV-infected mice is triggered by virus-specific CD4+ T cells targeting virus, and T-cell responses to multiple myelin autoepitopes may play a pathologic role in chronic disease (Miller et al., 1997).

3. Stress

3.1. The development of the concept of stress

Stress is an ever-changing, evolving concept since it was first introduced in the field of biology and medicine. In 1930s, Dr. Hans Selye first described general adaptation syndrome (GAS) when he injected rats with various impure and toxic gland preparations. He observed a triad syndrome, which includes enlargement of adrenal cortex, atrophy of thymus and lymph nodes, and gastrointestinal ulcers. The same responses were also observed when animals were exposed to other stimuli and toxin (Selye, 1936). According to his theory, GAS has three stages: the alarm, resistance, and exhaustion stages. When an organism is confronted with stress, the immediate response is alarm-the adrenocortical secretion increased with general sympathetic arousal. The resistance stage begins following the alarm reaction and is characterized by decrease in adrenocortical secretions and a return to normal body functioning. The exhaustion stage will follow if stimuli persist. Adrenocortical secretions rise again and may be totally depleted and eventually lead to “exhaustion”, even death (Selye, 1976). Selye “create a neologism and introduce the word ‘stressor’ for the causative agent” and introduced the word “stress” from physics for the resulting condition (Selye, 1974).

Chrousos proposed four key concepts that are closely associated with the comprehensive understanding of stress: *homeostasis, stressor, stress* and *adaptive response*. *Homeostasis* is described as an essential, complex dynamic equilibrium for maintaining life, and is constantly challenged by a variety of intrinsic and extrinsic adverse stimuli or *stressor*, which could potentially disrupt the equilibrium; *stress*, is referred to as a state of threatened homeostasis, either real or implied, which is reestablished by physiological and behavioral *adaptive response* (Chrousos, 1998).

McEwen introduced the terms "*allostasis*" and "*allosatic load*" into stress research. *Allostasis* is an essential component of maintaining homeostasis and an active process of adaptation by production of various mediators such as adrenal steroids, neurotransmitters, cytokines and tissue mediators. *Allostatic load* refers to the wear and tear on the body and brain resulting from chronic overactivity or inactivity of physiological systems that are normally involved in adaptation to environmental challenge. *Allostasis* emphasizes on the relatively large range of activities, functions and the duration of all the body systems in response to internal and external demands (McEwen, 1998).

Compared to the homeostatic mechanism, allostatic regulation (*allostasis)* is a broader and dynamic, rather than a set-point concept, which is also consistent with Selye's "alarm" stage. *Allostatic load* can be interpreted as the cost of adaptation (*allostasis*), the price of adaptation to adversity. The systems involved in the stress response or adaptation can be either rapidly mobilized and turned down or reactivated when necessary while dealing with stress (like Selye's resistance), and body remains in a relatively good state. In some cases, when they are not able to shut off or turn down these systems in response to stimuli, the excessive response will do harm to health (a

mechanism of autoimmune disease); or the inability to mount a response, the stressor will take over the body systems (a mechanism for infectious disease). Both situations produce a load on the body, because the normal protection afforded by these systems is lacking or exhausted (as in Selye's theory).

3.2. Stress response and individuals

The determinants of stress responses basically rely on two aspects: how the individual interprets stressor and the conditions of the individual when challenged by a stressor (McEwen, 1998).

1) Interpretation of stress by individuals varies among the population. The situation of threat may be viewed as a true stressor by some people, whereas it may not be perceived as a threat by other people. Quantitative genetics revealed that about two-thirds of reliable variance in measured personality traits are inherited (Chrousos, 1998). In addition, it is conceivable that biological and psychological maturity, social experience, and early training of an individual can affect how individuals interpret the situation, such that an older, matured and socially experienced person will more likely adapt to the stress, while the younger, immature and inexperienced individual may exhibit increased vulnerability to maladaptive stress response.

2) Conditions of the body. Obviously, people who are healthy or in good physical condition can more easily handle strenuous stress than those that are not in shape. Nevertheless, people who are already in a pathological state or at a critical state, will have an increased vulnerability to stress or/and exacerbation of disease (McEwen, 1998). For example, individuals suffering from long-term hypertension and hyperglycemia

(metabolic imbalance as a stressor) are more likely to develop type II diabetes.

3.3. Stressor and stress response

Stressor or stimuli include pathogens (bacteria, viruses, parasites etc.), toxins, irradiation, and various physical stimuli such as surgery and muscular exercise. Additionally, Cannon recognized the importance of the psychological response during stress (reviewed in Pacak and Palkovits, 2001). Currently, stimuli can be divided into four groups: 1) physical stressors that include cold, heat, noise and radiation. 2) psychological stressors, such as anxiety, fear or frustration. 3) social stressors, including dominance of animal, unemployment and divorce in humans. 4) stressors that challenge cardiovascular and metabolic homeostasis, such as exercise, orthostasis, heat exposure, hypoglycemia and hemorrhage (Pacak and Palkovits, 2001).

The body responds both behaviorally and physiologically to challenge by these stressors by mobilizing and incorporating endocrine, nervous and immune systems to maintain health state (homeostasis). In fact, almost all the body systems could be involved in the response. Behavioral adaptation includes improved cognition, focused attention, enhanced arousal, alertness and vigilance increased analgesia, elevations in core temperature, as well as concurrent inhibition of vegetative functions, such as appetite, feeding, and reproductive function. Concurrently, physical adaptation changes are primarily to promote an adaptive redirection of energy, such that oxygen and nutrients are shunted to the sites where they are needed the most, the CNS and the stressed body sites, by increasing cardiovascular tone (heart rate, cardiac ejection fraction, blood pressure), respiratory rate, and intermediate metabolism (gluconeogenesis, lipolysis) (Chrousos,

1998). If the adaptive response works in concert and is timely and adequate, health will be attained and the chance of survival will be increased; otherwise, either inadequate or excessive and prolonged response will lead to a disease.

3.4. The stress system

The stress system consists of central and peripheral limbs. The central part includes corticotropin-releasing hormone (CRH) neurons located in the paraventricular nuclei (PVN) of hypothalamus and paragigatocellular nuclei of medulla, and arginine-vasopresin (AVP) neurons of the hypothalamus, as well as catacholamine neurons of the locus ceruleus (LC) in medulla and pon. The peripheral stress system is composed of hypothalamus-pituitary and adrenal (HPA) axis and sympathetic nervous system (SNS) axis (Chrousos, 1998). The two systems receive information from the higher center of CNS and environment to produce the stress response.

When an organism encounters stress, both axes are activated. CRH secretion is increased, and is followed by elevation of ACTH from the pituitary and glucocorticoids (GCs) from adrenal cortex. GCs are the final effectors of HPA axis and exert their pleotropic effects on target tissues. CRH, ACTH and GCs interact with hypothalamic-gonadal axis by inhibiting gonadotropin hormone releasing hormone (GnRH), resulting in decreased production of luteinizing hormone (LH) and testosterone in males or estrogen in females and down-regulation of reproductivity. The activation of HPA axis also suppresses the secretion of growth hormone (GH) and insulin-like growth factor-1 (IGF-1) via inhibitory somatostatin (STS) stimulated by CRH. This alteration in growth axis may contribute to the development of psychological dwarfism, which is manifested

by delayed puberty due to emotional deprivation or psychological harassment during childhood or adolescent stage. Hypofunction of the thyroid can be induced by stress activated HPA axis. STS suppress the secretion of hypothalamic thyrotropin releasing hormone (TRH) and pituitary thyrotropin stimulating hormone (TSH). TSH, the synthesis of T4 and the conversion of inactive thyroxine (T4) to active triidothyronine (T3), are inhibited by GCs. Thyroid function profoundly influences other organ functions, such as on cardiovascular system and metabolism. Moreover, high GCs, GCs-induced low GH and/or IGF-1, as well as cytokines induced by stress, may further interfere with metabolism, resulting in gluconegenesis (hyperglycemia and insulin resistance) and visceral adiposity (Chrousos, 1998).

Compared to the HPA axis, the SNS provides a more rapid response that mostly controls the acute response to a stressor, as well as a wide range of functions. Sympathetic innervations of these peripheral organs are derived from the efferent preganglionic fibers, whose cell bodies are lined at the intermediolateral column of the spinal cord. The nerve fibers of the sympathetic postganglionic neurons innervate vascular smooth muscle, skeleton muscle, kidney, gut, fat and many other tissues and organs. Activation of SAM results in the secretion of norepinephrine (NE) and epinephrine (Epi), acetylcholine (Ach) as well as neuropeptide Y (NPY), STS, enkephalin and nitric oxide (NO) etc. Additionally, circulating NE and Epi also comes partly from humoral secretion by the adrenal medulla during stress. SNS, together with the HPA axis, participates in the regulation of adaptation to the stressor in reproduction, growth and metabolism as described above. Furthermore, the SNS plays a role in gastrointestinal function during stress. Activation of the SNS enhances sacral parasympathetic neural

activity, thus increasing colonic motility; it also inhibits gastric motility, gastric acid secretion, empting of the stomach, with the assistance of CRH. Therefore, stressed people may exhibit chronic gastrointestinal pain and irritable bowel (Elenkov et al., 2000).

3.5. The interaction between behavior, brain and immunity

Two potential pathways are known for the connection between the nervous system and immune system. First, immune organs, such as the thymus, spleen and bone marrow are innervated by sympathetic nervous system via catecholamines, while the immune cells carry catecholamine receptors. Another pathway is mediated by hormones released from the nervous system and endocrine system. Likewise, the immune cells contain receptors for these hormones, such as GCs. In contrast, the immune cells can secrete soluble molecules, such as cytokines, which act on the nervous system through receptors. Thus, a bidirectional communication is established for the regulation between the two systems in response to a variety of stimuli, for example, stress (Maier et al., 1994; Prutte, 2003).

4. Stress and multiple sclerosis

The linkage between stress and the exacerbation of MS was first noted by Charcot, one of the earliest investigators of MS, in his publication “Lectures on the Disease of the Nervous System” in 1877. In his book, the emotions of grief, vexation, and adverse changes in social circumstances were attributed to MS. The phenomenon has been noticed by other investigators and since that time, the potential association between psychological stressor and clinical onset or exacerbation has been investigated based on

case-control and longitudinal studies (reviewed in Mohr and Cox, 2001).

It has been shown that chronic psychological stressors, such as interpersonal conflicts, low perceived social support, anxiety and depression episodes, are potential risk factors for the exacerbation of MS (Strenge, 2001). A significant positive correlation was also suggested between postpartum emotional distress and MS symptoms in mothers with MS (Gulick and Kim, 2004). Moreover, it was observed that stressful life events usually precede the exacerbation of MS in relapsing-remitting MS patients (Ackerman et al., 2002; Bach and Wild, 1990). The most complete data to date was reported by Mohr et al. (2004) using a meta-analysis on 14 previous studies, in which the data sources were related with "stress", "trauma" and "MS". The results strongly suggest an association between stressful life events and MS exacerbation. The relationship between stress and MS exacerbation was further supported by MRI scans, which showed that stress was significantly correlated with the development of new gadolinium enhancing (Gd+) brain lesions, 8 weeks after stress (Mohr et al., 2002).

However, the notion that stress may exacerbate MS has been controversial due to inconsistent observations. For example, a study conducted in Israel during the Gulf War documented a lower frequency of relapses in MS patients exposed to the threat of missile attacks (Nisipeanu and Korczyn, 1993). Sibley (1997) noticed clinical exacerbation following martial and job-related stress, but not negative life events, such as a death in the family. This indicates that different types of stressor may impact the disease course differently. Others argue that the studies have a critical limitation in "recall bias", so that the patients may be more likely to recall such events as stressful episodes when searching their memories for associations between stressor and MS attack (Galea et al., 2004). In

addition, MS patients display psychological symptoms, such as depression, anxiety and anger (Mohr and Cox, 2001). Therefore, "psychological stress" and neurological relapse may be different temporal manifestation of the same disease process (Galea et al., 2004).

The conundrum has led to further research on the alteration in immune and endocrine parameters of MS patients following stress. Heesen et al. (2002) reported elevated baseline levels for catecholamines, prolactin and IL-6 in relapsing-remitting MS patients as compared to healthy controls, suggesting an altered stress hormone system in MS, a deficiency in regulatory feedback mechanisms; and a marginal hyporesponse of TNF-α and IL-10 was noted as well (Heesen et al., 2003). Furthermore, Ackerman et al. (2003) noted that MS patients with higher cardiovascular reactivity to stressors and higher baseline heart rate demonstrated increased frequency in exacerbations, indicting a potential role of autonomic tone and stress reactivity in stress-related exacerbations. Although the current literature is limited, it is becoming one of the most prospective areas in MS investigation. Unquestionably, stress is regarded as one of the triggering factors in the pathogenesis of MS.

5. Stress and infectious disease

5.1. Early research on the relationship between stress and infectious disease

The stress effect on the progression of disease has been noticed for decades. Most of the observations were based on longitudinal or retrospective studies, which were less convincing and more at descriptive level. In the early 1990s, Cohen et al. (1991) established experimental methodology in humans for the exploration of the relationship between stress exposure and the susceptibility to infectious disease, which led to the

boost in the development of a new interdisciplinary area – psychoneuroimmunology (PNI). Recently, the impact of stress on the infectious diseases caused by viruses, bacteria, and parasites has been extensively investigated by dissecting immune parameters at both the cellular and molecular levels.

Upper respiratory infections are very common in humans with a higher incidence in persons under stress. In a study of 394 volunteers conducted by Cohen et al. (1991, 1993), all the healthy subjects were given nasal drops containing one of five common respiratory viruses (rhinovirus type 2, 9, or 14, respiratory syncytial virus, or coronavirus type 229E) after completing questionnaires assessing degrees of psychological stress, and then the subjects were quarantined and monitored for the development of evidence of infection and symptoms. It was demonstrated that stressful life events, perceived stress and negative effect predicted a linearly greater probability of developing a cold across the five different viruses. In the following experiment, Cohen et al. (1998) identified different types of stressor impact on the susceptibility at various degrees. He found that severe chronic stressors (1 month or longer) were associated with an increased risk of developing colds after inoculation with common cold viruses; whereas severe acute stressful life events (less than 1 month long) were not. He also noted that this relation was attributable primarily to under- or unemployment and to enduring interpersonal difficulties with family or friends. Although the differential susceptibility to the common cold could not be explained by differences among stressors, these experiments avoided methodological pitfalls such as “recall bias” and gave rise to convincing evidence of the link between stressful life events and susceptibility to infectious disease.

The Herpesviridae family includes eight viruses that may cause acute and recurrent

disease. Herpes simplex virus type-1 (HSV-1) causes encephalitis, keratitis and stomatitis; and herpes simplex virus type-2 (HSV-2) is the pathogen for genital herpes. Many studies have been focused on the relationship between stress and recurrent herpes infection. Hoon and colleagues conducted a study of 125 college students who were seropositive for genital herpes, and found that psychosocial stressor increases herpes recurrence, as well as the vulnerability to nonherpes, and general illness (Hoon et al., 1991).

The role of stress and negative effect on the progression of HIV infection has been investigated. It was shown that depression predicted a more rapid decline in $CD4^+$ T cell counts and this association was not attributable to baseline physiological difference (Burack et al., 1993). However, in another study, Kessler et al. (1991) showed that stressful life events did not correlate with T cell count and disease course. This contradiction, in fact, may reflect the inconsistencies in the assessment or quantitation of the stressor. It was greatly reconciled with the establishment of animal models for the study of stress, which allowed conceptualizing and measuring the stressor, and by examining immune parameters, shedding light on the underlying mechanisms that mediate the interaction between stress and susceptibility of disease.

5.2. Experimental models for stress

Restraint stress, or immobilization, is a stressor that limits the movement of animals to induce stress response. It was originally used as a basic technique for the investigation of pharmacological, physiological or pathological phenomena or with restraint stress itself serving as the object of the study. Recently, researchers extended the application of restraint stress to investigate CNS mechanisms in peripheral disease. Since then, the

restraint technique has evolved as a standard laboratory procedure for studying stress, so that restraint has become a synonym of stress (Glavin et al., 1994). Basically, the restraint procedure involves confining the animal in a cylindrical, plastic tube for time periods ranging from minutes to hours. The stress response was validated by observing an increase in plasma corticosterone and cortisol secretion following restraint stress in primates and rodents; however, a slightly different response was noted in rats and mice (reviewed in Glavin et al., 1994). Murison and Overmier (1993) reported that restraint induced gastric and duodenal ulcerations in conscious rats, but not in unconscious rats, suggesting the involvement of CNS in the stress response. Restraint stress has been used as a valuable tool mimicking psychological stressors, such as anxiety or distress, in the study of stress.

Social disruption is another stress procedure by introducing an animal (intruder) into another animal (resident)'s territory to produce confrontation response. This procedure is based on the establishment of a territory, usually by a male animal, and defense against an unfamiliar male intruder (Stefanski et al., 2003), thus disrupting the established hierarchy in the territory, which generalizes in human as social distress. Maternal separation stress is evoked by taking the baby animals away from their mother, produces offspring with behaviors reminiscent of the cardinal features of anxiety and affective disorders. This stress paradigm also produces persistent hyperresponse in hypothalamic-pituitary-adrenal axis activity secondary to hypersecretion of corticotropin-releasing hormone and is used as laboratory tool for psychological stress studies (reviewed in Newport et al., 2002).

Other stress models, such as electric foot shock, applying electric stimuli via the foot

of the animal; swimming stress, by placing the animal in a "swimming pool"; cold or thermal stress, by exposing the animal in a cold (15-20^{0}C) or heat (38-39^{0}C) environment, as physiological and psychological stressors, are also applied in the study of stress and infectious disease.

5.3. Stress increases the susceptibility to infectious disease by suppressing immune function

The stress-induced viral pathogenesis has been extensively investigated using a restraint stress model with influenza and herpes simplex virus infections. It was shown that both HPA and SNS axes are activated following restraint stress evidenced by increases in plasma glucocorticoids (GC) and catecholamines (Hermann et al., 1994a & b). Influenza-infected mice, when subjected to restraint stress display marked a decrement in the immune response to viral infection, including decreased NK cell activity, which is mediated by GC and opiods and a delayed recruitment of cells to the lung (Hunzeker et al., 2004; Tseng et al., 2004); decreased infiltration and pro-inflammatory cytokine response (Konstantinos and Sheridan, 2001; Sheridan et al., 1991; Sheridan et al., 2000) in the lung. A series of gene microarray expression experiments in this restraint and influenza model showed a reduced and delayed expression of genes that are responsible for cell adhesion, cell trafficking, MHC and antigen presentation, and chemotaxis (Engler et al., 2005). These results demonstrate a significant suppressive effect of restraint stress on the antiviral immunity to influenza, which illustrates at the cellular and molecular level, the mechanism behind increased susceptibility to common cold in stressed humans. In addition, restraint stress has a greater suppressive effect on

aged mice than young mice (Padgett et al., 1998a), which correlates with a greater vulnerability to infection in elders.

Furthermore, the stress-induced effect on HSV-1 infection has been studied using a restraint stress model. NK cell and HSV-specific CTL were depressed by restraint stress (Bonneau et al., 1991a; Bonneau et al., 1993), and both the HPA and SNS axes contributed to the suppressive effect (Dobbs et al., 1993). In this model, restraint stress was shown not to inhibit the generation of virus-specific memory CTL (CTLm), but it did suppress CTLm by inhibiting the activation of CTLm to become the lytic phenotype (Bonneau et al., 1991a), or proliferation of CTLm as a result of decreased IL-2 production in restrained mice (Bonneau et al., 1991b) in lymph nodes. Splenic HSV-specific CTLm was also reduced, accompanied by a decrease in cytokine production following restraint stress, suggesting that a reduced availability of cytokines which drive the activation of CTLm may be responsible for the suppressed CTLm function (Bonneau, 1996). As a neurotropic virus, the consequence of restraint stress on the CNS infection was investigated. It was demonstrated that stress causes severe HSV encephalitis and higher mortality, and decreased cellularity (DeLano and Mallery, 1998) and a delayed HSV-specific $CD8^+$ infiltration (Anglen et al., 2003) in the CNS. Additionally, restraint stress decreases the clinical severity of primary, local HSV-1 infection in the skin by down-regulating interferon production (Ortiz et al., 2003). Thus, stress-induced dysregulation appears to be systemic in HSV infection, not only limited to local infection. This model provides us another example illustrating the mechanisms by which stress increases the incidence of infection.

However, the reactivation of HSV infection was not induced in this herpes-restraint

stress model; yet disruption of the social hierarchy within colonies of mice increased aggression among cohorts and caused reactivation of latent HSV-1 in 40% of latently infected animals (Padgett et al., 1998b). Therefore even though the HPA axis is activated in both models, the outcomes are different. Likewise, the differential outcomes on the antiviral response by the two models were also observed in the influenza model. Sheridan et al., (2000) documented a diminished infiltration in the lung by restraint, but an increase by social disruption. This is due to the elevation in circulating nerve growth factor (NGF) released by social disruption, which may negatively regulate the expression of type II glucocorticoid receptors and hence the induction of steroid insensitivity. It was also recorded that exposure of mice to immobilization stress and subsequent inoculation of influenza virus does not significantly influence gastric ulceration, and cold stress resulted in a decrease in the index of stomach ulceration. However, the simultaneous application of cold-restraint stress and influenza virus infection exacerbated ulceration (Mileva et al., 2003). It is conceivable that the intrinsic machinery involved in regulating the stress response varies in different stress paradigms; the virus-induced immune response also differs due to the properties of virus. Therefore, it is always important to choose a proper model in order to uncover the mechanism of the interaction between stress and infectious disease.

In order to investigate the effect of stress on pathogenesis of MS, we utilized a restraint stress model mimicking anxiety and distress, which are common psychological stressors in MS patients; and a Theiler's virus model, which is based on the hypothesis that virus is a potential etiological agent of MS. A previous study has shown that restraint stress results in higher mortality, increase in circulating corticosterone, lymphopenia

(Campbell et al., 2001) and decreased NK cell activity (Welsh et al., 2004). In this current study, we continued to study the acute infection by further dissecting pro-inflammatory responses following stress, and we explore how stress impacts the development of demyelination in the chronic disease. We hypothesize that stress-induced immunosuppression raises the susceptibility to acute viral infection, thus resulting in persistent virus infection and facilitation of demyelination.

II THE EFFECT OF RESTRAINT STRESS ON THE CLINICAL SIGNS, CNS INFLAMMATION AND CHEMOKINE EXPRESSION DURING THE ACUTE TMEV INFECTION*

1. Introduction

In recent decades, accumulative data from both clinical and experimental observations have indicated that stressful life events may be potential risk factors contributing to the onset and progression of autoimmune diseases such as multiple sclerosis (MS) (Ackerman et al., 2002; Grant et al., 1989; Warren et al., 1982; Warren et al., 1991). Exposure to physical or/and psychological stressors can result in the exacerbation of clinical signs in MS patients, such as an increase in degree of disability, worsening of cognitive difficulties and an increase in severity of brain lesions (Mohr et al., 2002; Zorzon et al., 2001). The mechanism by which stress regulates susceptibility and severity of autoimmune diseases has been extensively investigated. The biological impact of stress is exerted via a feedback regulatory loop within a network consisting of the central nervous system (CNS), the endocrine system and the immune system. The stress response is initiated in the CNS by the limbic-hypothalamic-pituitary adrenal axis, and by the locus coeruleus-norepinephrine autonomic system. The stress-induced release of hypothalamic corticotropin-releasing hormone (CRH) ultimately results in the production of adrenal glucocorticoids. Additional activation of the sympathetic nervous

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system (SNS) causes the production of catecholamines. The end-point products from both systems further affect host defense and the immune system.

Behaviorally, restraint stress (RS) causes anxiety and hyperactivity, followed by lethargy (Faith et al., 1999). RS has frequently been used experimentally as a stressor to examine the influence of stress on the immune response. Previous studies have revealed that RS can have a profound influence on the immune responses by increasing the susceptibility to viral infection, delaying virus-specific antibody production in influenza-infected mice, suppressing NK cell and herpes simplex virus (HSV)-specific cytotoxic T lymphocytes activities and by prolonging wound-healing time. (Zwilling et al., 1990; Bonneau et al., 1991 and 1993; Sheridan et al., 1991; Padgett et al., 1998).

Intracerebral inoculation of Theiler's murine encephalomyelitis virus (TMEV) produces a biphasic disease consisting of an acute polioencephalitis in all strains of mice and chronic inflammatory demyelination in genetically susceptible strains of mice (Theiler, 1934; Lipton, 1975). Theiler's virus-induced demyelination (TVID) has proved to be a valuable experimental animal model for the study of multiple sclerosis. The acute infection normally lasts approximately two weeks and the virus preferentially infects neurons within gray matter. In the later demyelinating phase, TMEV predominantly persists in CNS macrophages/microglia and in oligodendrocytes and astrocytes to a lesser extent, and is accompanied by flaccid paralysis and demyelination. Host responses to Theiler's virus during early infection are critical in determining either successful viral clearance or persistence of virus in CNS with the development of demyelination. Inflammation is mediated by virus specific $CD4^+$ T cells targeting infected CNS-resident antigen presenting cells (APCs), and is followed by activation of myelin-specific

autoreactive $CD4^+$ T cells via epitope spreading and myelin reactive antibodies, which eventually results in demyelination (Miller et al., 1997; Welsh et al., 1987 and 1989; Borrow et al., 1998). T cells are initially activated peripherally in lymphoid tissues following infection and then traffic back into the CNS to effect viral clearance (reviewed in Tompkins et al., 2002).

Recruitment of inflammatory cells into the CNS is a crucial step in establishing the host's defense and ultimately viral clearance. Chemokines are a family of low molecular weight (8-10 kDa), inducible, secreted, pro-inflammatory cytokines that serve as potent chemoattractants for immune cells in response to a myriad of immune and inflammatory responses. Additionally, they are important regulators of leukocyte recruitment, trafficking and activation. To date, over 40 chemokines are classified into four subfamilies (C-X-C, C-C, C, C-X_3-C) according to the configuration of cystein residues near the N-terminal. C-X-C chemokine, also known as α chemokine, include GRO (growth-related oncogene), PF4 (platelet factor 4), IL-8 (interleukin 8), Mig (monokine induced by interferon γ), IP-10 (interferon-inducible protein 10), BCA-1 (B-cell-attracting chemokine 1), etc. α chemokines are chemotactic mainly for neutrophils and lymphocytes; C-C or β chemokines preferentially attract monocytes, eosinophils, basophils and lymphocytes, over 20 chemokines belong to this subfamily, such as MCP-1,2,3,4 (monocyte chemoattractant protein 1,2,3,4), MIP-1α,β (macrophage inflammatory protein 1α and β), RANTES (regulated on activation, normal T-cell-expressed and –secreted), Eotaxin; C consist of two chemokines: lymphotactin/SCM-1α (single C motif-1α) and SCM-1β. The only member of C-X_3-C chemokine family is fractalkine (available at http://www-ermm.cbcu.cam.ac.uk/02005318a.pdf).

It has been suggested that chemokines are involved in T cell (both $CD4^+$ and $CD8^+$)-mediated viral clearance following viral infection, such as Theiler's virus, mouse hepatitis virus and herpes virus etc. (Theil et al., 2000; Hoffman et al., 1999; Murray et al., 2000; Melchjorsen et al., 2002; reviewed by Glass et al., 2002). The role of chemokines in one of the autoimmune diseases, experimental autoimmune encephalitis (EAE), has been extensively studied. A broad profile of chemokines consisting of MCP-1, RANTES, IP-10 and MIP-1α was detected in the CNS (e.g. Sorensen et al., 1999; reviewed by Karpus, 1998; Eng et al., 1996); moreover, the chemokine patterns exhibit temporal and spatial difference with respect to the progression and activity of disease and lesion development (see reviews by Karpus and Ransohoff, 1998). This phenomenon has also been observed in the biphasic phase of TVID (Hoffman et al., 1999). In addition, a recent clinical study has demonstrated an up-regulation of IP-10 in cerebrospinal fluid (CSF) of patients with relapsing-remitting multiple sclerosis, which was correlated with lesion activity as detected by MRI scans (Mahad et al., 2002; Sindern et al., 2002). Furthermore, neutralizing antibody against certain chemokine or chemokine receptor antagonist proved effective in reducing both the accumulation of inflammatory cells and in ameliorating clinical signs of disease (Fife et al., 2001; Karpus et al., 1995; reviewed by Ransohoff et al., 2000). Taken together, these data implicate the involvement of chemokines in the pathogenesis of MS.

We have previously shown that RS increases the mortality during the acute stage of Theiler's virus infection by corticosterone-induced immunosupression and impaired viral clearance mechanisms (Campbell et al., 2001; Welsh et al., 2004). It has also been demonstrated that stress may impact migration or redistribution of lymphocytes between

blood and lymphoid organs (Stefanski, et al., 2003; reviewed by Dhabhar, 2002). In the current study, we further investigated the mechanism involved in restraint stress-induced alteration of the immune response and lesion development in both the CNS and peripheral lymphoid tissue following Theiler's virus infection. This was accomplished by examining the alterations in chemokine expression in both the brain and spleen of restrained and non-restrained CBA mice. We demonstrate that restraint stress down regulates the expression of certain chemokines and diminishes immune cellular infiltration into the CNS.

2. Materials and methods

2.1 Mice

Three-week-old male CBA mice (purchased from Harlan Labs, Indianapolis, IN) were housed in pathogen-free condition. The mice were housed 3-4 mice per cage, counterbalanced by weight upon arrival, and acclimated for one week prior to commencing the experiments.

2.2 Virus

The BeAn strain of Theiler's virus was obtained from Dr. H. L. Lipton, Department of Neurology, University of Chicago, Evanston, IL and was propagated and amplified in L-2 cells. The culture supernatant containing infectious virus was aliquoted and stored at -80^{o}C before use (Welsh et al., 1987).

2.3 Experimental design

A 2 (infected vs. non-infected) by 2 (restraint vs. non-restraint) factorial design was employed. At four weeks of age, cages were randomly assigned to one of the following treatment conditions: (1) Non-infected/Non-restrained (NI/NR) mice remained undisturbed in their home cages; (2) Non-infected/Restrained (NI/R): each mouse was subjected to restraint stress in their home cages; (3) Infected/Non-restrained (I/NR): mice were infected intracerebrally with BeAn virus; or (4) Infected/Restrained (I/R): each mouse was intracerebrally infected with virus and subjected to restraint stress.

Three experiments were conducted in this study in order to evaluate chemokine alterations and CNS lesions. In each experiment, the mice were divided into four groups as described above. The specific procedure followed plus numbers of mice used per group (in parenthesis) were: 1) To determine chemokine alterations at day 2 p.i.: NI/NR (4), NI/R (4), I/NR (4), I/R (5); 2) To determine chemokine alterations at day 7 p.i.: NI/NR (8), NI/R (8), I/NR (7), I/R (14); 3) To determine microscopic lesions in CNS: NI/NR (3), NI/R (3), I/NR (3), I/R (6).

2.4 Restraint stress protocol

The restrained mice were placed in well-ventilated restraining tubes (2.7-cm internal diameter and 14-cm length) for 12 h (from 9pm-9am) each of 3 or 7 consecutive nights (Sheridan et al., 1991; Campbell et al., 2001). The tubes were perforated with small holes to allow adequate ventilation. Restraint stress was initiated 1 day prior to infection and continued for 2 or 6 subsequent sessions (for the mice sacrificed at day 2 p.i. and day 7 p.i., respectively) as indicated above.

2.5 Infection of mice

Mice were anesthetized with metofane and then either intracerebrally inoculated into the right cerebral hemisphere with a $5x10^4$ plaque forming unit (PFU) of BeAn viral inoculum in a 20µl volume (infected mice) or sham-infected with 20µl of sterile phosphate-buffered saline (PBS) (non-infected group).

2.6 Behavioral assessment

All mice were weighed at the beginning of the experiment and daily 3 hours after the stressed mice were release from restraining tubes. Daily food intake per cage was also recorded. Mice were scored daily for clinical signs of disease according to the following criteria: 0=healthy, grooming complete, active; 0.5=fur slightly ruffled, grooming incomplete; 1=ruffled fur and/or slightly hunched posture; 2=hunched, slightly lethargic or irritable; 3=very lethargic, unresponsive, very hunched, sunken eyes; 4=dead.

2.7 Spontaneous activity monitoring

Spontaneous activity has been shown to be a sensitive measurement indicative of demyelination in Theiler's virus infection (McGavern et al., 1999; Sieve et al., 2004; Yirmiya et al., 1994). To monitor the activity, individual mice were placed in each of four Digiscan monitors consisting of a 20 × 20-cm clear Plexiglas box with a grid of horizontal infrared beams mounted every 2.5 cm. Two tiers of beams were mounted 2 cm and 3 cm above the floor. The four monitors were connected to a Digiscan Analyzer (Omnitech Model DCM-8, Columbus, OH), as shown in an illustration (Figure 2-1), for translation of the activity data to a computer.

The number of beam interruptions was recorded and analyzed by Digiscan for assessment of activity in each 20 minute session. The following variables, either calculated directly by the Digiscan Analyzer or derived from the measured variables, were examined:

1) Total distance (TD). The horizontal distance traveled by an animal in a session.

2) Horizontal activity (HA). The number of separate horizontal movements executed by an animal with a minimum stop time of 1 s between movements.

3) Vertical activity (VA). The number of separate vertical movements (rearing) in a session, separated by at least 1 s.

4) Central time (CT). The total time a mouse spent in the movement at the central area in a session.

5) Center distance (CD). The distance traveled by a mouse in the central area in a session.

6) Marginal time (MT). The amount of time a mouse spent in the movement at the marginal area.

7) Marginal distance (MD). The distance traveled by a mouse in the marginal area in a session.

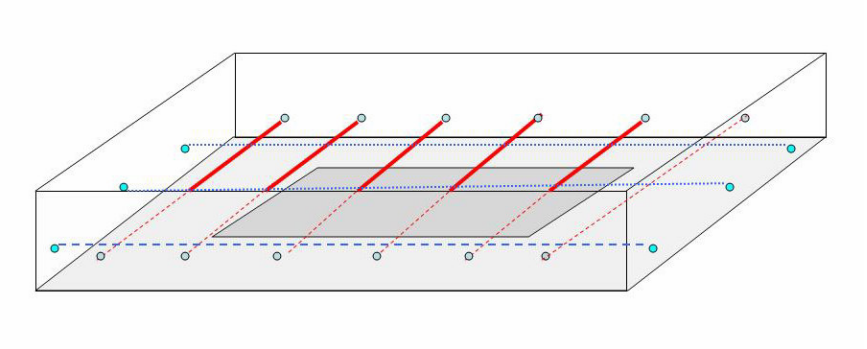

Figure 2-1 An illustration showing one of the 4 digiscan monitors of an activity monitoring box. A representative tier of infared beam was shown. The highlighted area indicate the central area, at which the activity and time a mouse spend during a 20 min monitoring session are recorded as central activity and central time. The rest region indicates the marginal area for recording marginal activity and marginal time of a mouse.

2.8 Termination of mice

Mice were sacrificed by pentobarbital injection i.p. at either day 2 p.i. or day 7 p.i. and then were perfused with diethyl pyrocarbonate (DEPC, Sigma)-treated PBS. The brains and spleens were removed, weighed, then were quick frozen in liquid nitrogen and stored at -80^{o}C until used.

2.9 Ribonuclease protection assay

Total RNA was extracted from the brain and spleen using TRIZOL Reagent (Life Technologies, Grand Island, NY) according to the manufacturer's instructions. The mCK-5c Multi-Probe Template Set (BD PharMingen, San Diego, CA) contains DNA templates for chemokines lymphotactin (Ltn), RANTES, MIP-1β**,** MIP-1α, MIP-2, IP-10, MCP-1, TCA-3 (T cell activation-3), Eotaxin, and two housekeeping genes L32 (ribosome RNA) and GAPDH (glyceraldehydes-3-phosphate dehydrogenase). The probe was labeled with ^{32}P –UTP (800 Ci/mmol, 10μCi/μl, PerkinElmer Life Sciences, Inc., Boston, MA). Ribonuclease protection assay (RPA) analyses were performed using RPA III and MAXIscript kit (Ambion, Austin, TX), according to manufacturer's instructions.

Briefly, 25μg RNA was hybridized overnight (16 h) with labeled probe and the reactions were subsequently digested with RNase A/T1 Mix, followed by precipitation with Inactivation/Precipitation III solution (Ambion, Austin, TX). The protected RNA-probe complexes were loaded and separated on 5% acrylamide/8M urea/1xTBE gel. Then the gel was dried for 2 h using a gel drier (Gel Drier, Model 583, Bio-Rad), and exposed to a Fujifilm Phosphor Imaging Plate in BAS cassette 2325 (Fuji Medical Systems USA, Inc.) for 16 h.

The plate was scanned with a Fujifilm Bio-imaging Analyzer BAS-1800II (Kanagawa, Japan) and the photo-stimulated luminescence (PSL) was analyzed with the ImageGauge software (Version 3.12, Science lab, Fuji Photo Film Co.Ltd). The densitometry of each band was represented by the PSL reading and then normalized with a housekeeping gene (L32 or GAPDH).

2.10 Microscopic examination of brain tissue

Brain samples were collected from mice in a second identical experiment at day 7 p.i. by terminal perfusion with 10% formalin. The brains were removed, processed and embedded in paraffin. Five-micron sections from each block were stained with hematoxylin and eosin (H&E) for microscopic examination.

Brain sections were scored for perivascular cuffing, meningitis, and microgliosis by a trained experimenter blinded to the subject's treatment condition. A micrometer was used to determine the measurements of circumference, area, and diameter. The score for perivascular cuffing was set by calculating the product of the average number of cell layers surrounding the vessels, the number of cuffs in the hippocampus, and the diameter of the cuff. The scoring for meningitis was determined by calculating the number of inflammatory cell layers in the meninges, multiplied by the length of the meninges affected by inflammation and divided by the circumference of the meninges of the section. Microgliosis was evaluated by the cell number of microgliosis in the hippocampus divided by the area of the hippocampus. The score for each animal was derived from the average of 2-3 sections.

2.11 Statistical analysis

Analyses of variance (ANOVA) were used to evaluate between group differences across stress, infection, and time of sacrifice conditions. These analyses were followed by post hoc group mean comparisons using Duncan's New Multiple Range Test. Results were considered significant if $p < 0.05$.

3. Results

3.1 The effect of restraint stress on behavioral signs during the early phase of TVID

3.1.1 Clinical score

As depicted in Figure 2-2, clinical scores were elevated in both the infected and non-infected restrained mice compared to the non-restrained infected and non-infected mice. The majority of the restrained mice (33/39) exhibited signs of ruffled fur and poor grooming on the second day of restraint stress (1 day p.i.) which became exacerbated with continued restraint. Some of the mice developed a hunched posture, but no signs of encephalitis in infected mice were seen at this early stage of disease. An ANOVA conducted on the worst clinical score observed over the 7 day period confirmed that there was a significant main effect of restraint stress, F (1,64) = 154.41, $p < 0.0001$. However, the main effect of infection, F (1,64) = 1.703, $p > 0.05$, and the interaction between restraint and infection were not significant, F (1,64) = 1.703 $p > 0.05$. A similar pattern was observed in a previous study during the first 7 days post infection; however, when restraint was continued for an additional 2-3 weeks, the infected/restrained mice developed severe signs of encephalitis and high mortality (Campbell et al., 2001). Thus, one week of restraint stress is too early to observe an interaction between restraint and infection on clinical signs of acute infection.

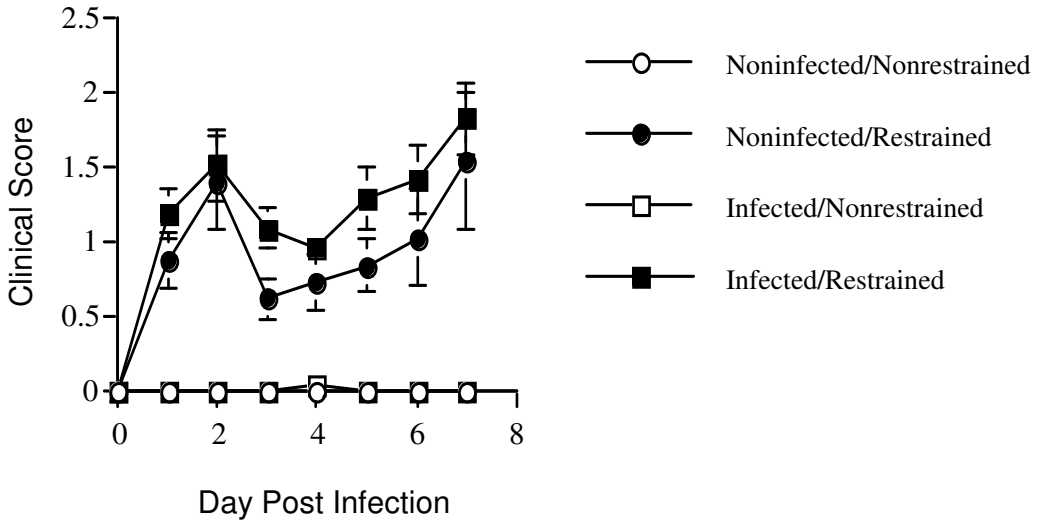

Figure 2-2. Clinical score following TMEV infection and restraint stress during the acute phase of TVID. Restraint stress exacerbates clinical scores in both infected and non-infected restrained mice during the first 7 days post infection. Non-restrained (both infected and non-infected) mice displayed no apparent clinical signs at this time point. Data are expressed as the mean $\pm$ SEM.

3.1.2 Spontaneous activity

The effect of restraint stress was also evidenced by spontaneous activity monitoring as depicted in Table 2-1 and Figure 2-3. There was a main effect of stress on center time (CT), center distance (CD) and marginal time (MT), all Fs > 8.623, all ps < 0.0097. The data demonstrated that the vertical activity (VA) was reduced, and the overall spontaneous activity declined in the central area but increased in the marginal area following restraint stress. A main effect of days post infection was found on total distance (TD) and center distance (CD), such that both total travel movement and the time spent in the central area in a session were reduced with time, all Fs > 6.237, all ps < 0.0238. No effect of infection on spontaneous activity was found. The interaction of restraint by day p.i. on horizontal activity (HA), center time (CT) and marginal time (MT) were present, such that an increase in HA, CT and a decrement in MT were observed in nonrestrained animals over time (from day 1 p.i. to day 4 p.i.); whereas the opposite, a decrement in HA and CT and an increase in MT were noticed in restrained mice with time (from day 1 p.i. to day 4 p.i.), all Fs > 4.847, all ps < 0.0427. These results suggested that the spontaneous activities of restrained mice were significantly decreased, so that the restrained animals spent more time in the margin and less time in the center, indicative of sign of anxiety following stress; and that a further reduction of such an effect of stress was seen with extended restraint stress.

Table 2 -1 The main effect of restraint stress and day postinfection on spontaneous activity variables

Measure	Stress		Day post infection	
	Non-restrained	Restrained	day 1 p.i.	day 4 p.i.
Total distance (TD)	611.43 ± 27.63	555.92 ± 38.81	639.40 ± 33.58 *	516.85 ± 35.00 *
Horizonal activity (HA)	2692.69 ± 64.36	2704.67 ± 102.01	2747.90 ± 94.42	2651.85 ± 92.61
Vertical activity (VA)	333.13 ± 22.58 *	212.58 ± 19.32 *	285.95 ± 22.02	235.65 ± 26.08
Central time (CT)	373.83 ± 40.48 *	196.28 ± 28.62 *	265.79 ± 29.80	268.82 ± 46.18
Central distance (CD)	262.63 ± 20.34 *	187.63 ± 16.16 *	262.63 ± 20.34 *	187.63 ± 16.16 *
Marginal time (MT)	826.17 ± 40.48 *	1003.72 ± 28.62 *	934.22 ± 29.80	931.18 ± 46.18
Marginal distance (MD)	348.56 ± 16.88	368.67 ± 26.35	390.00 ± 25.08	331.25 ± 21.89

* denotes significance between two conditions (restrained vs. non-restrained or day 1 p.i. vs.day 4 p.i.), when $p < .05$. The data was represented by mean±S.E.M.

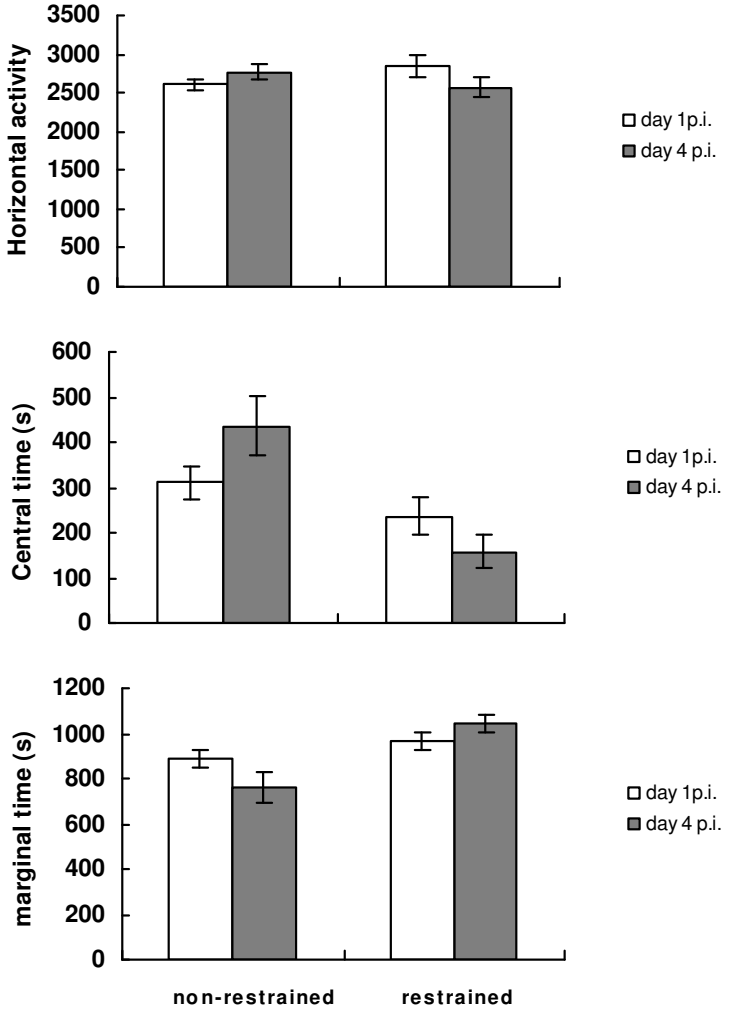

Figure 2-3. The effect of restraint stress by day post infection interaction on spontaneous activity variables. The effect of restraint stress on reducing horizontal activity (HA) was not shown until day 4 p.i.. Restraint stress diminished the time a mouse spend in the central area (CT), whereas increased the marginal time (MT). The change in time of movement at different area was more evident with extended restraint stress, as revealed by ANOVA, all Fs > 4.847, all ps < 0.0427.

3.1.3 Body weight

Body weight was dramatically reduced over time by restraint stress in both the infected and non-infected mice (Figure 2-4). Again, an ANOVA confirmed a significant main effect of restraint, F (1,231) =76.143, $p < 0.0001$, and time of sacrifice, F (7,231) = 5.251, $p < 0.0001$; however, neither the main effect of infection nor its interaction with restraint or time were significant, all Fs (1,76) <0.403, ps >0.53. It is noteworthy that we observed black feces in one non-infected/restrained and two infected/restrained mice at necropsy. However, no evidence of hemorrhage or ulcers was evident in the stomach or intestine of these mice when examined histologically (data not shown).

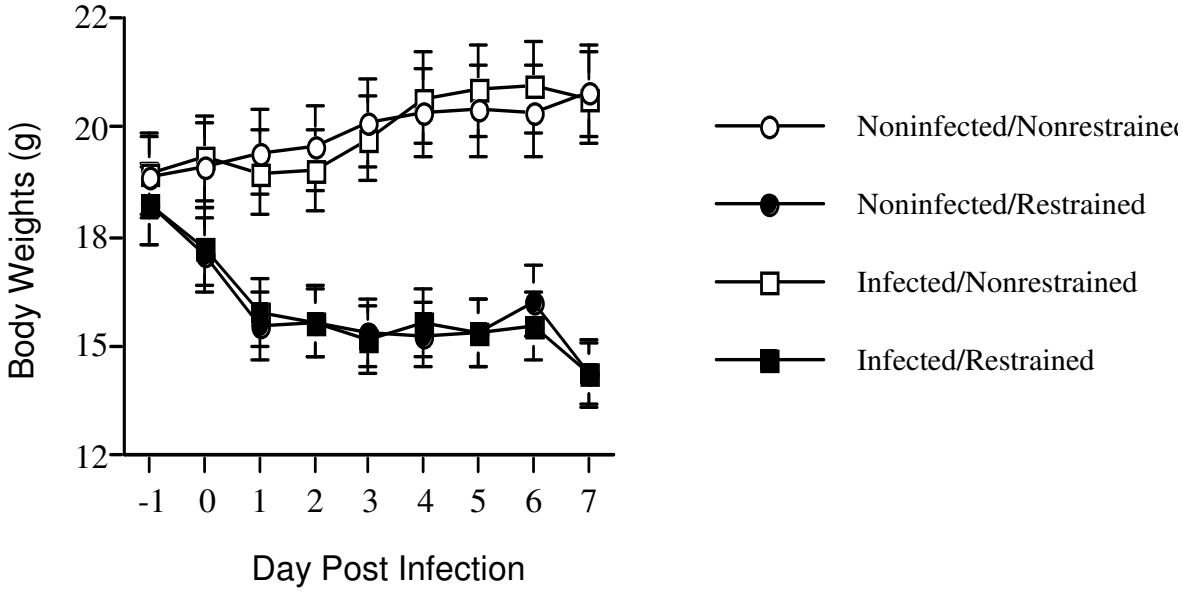

Figure 2-4. Effect of restraint stress on body weight. Restraint stress caused loss of body weight in both infected and non-infected mice. Data are expressed as the mean $\pm$ SEM.

3.2 Effects of stress on spleen and thymus weights

The physiological impact of the restraint stress manipulation was also reflected by reductions in organ weights when mice were sacrificed at day 7 p.i. (Figure 2-5). Restraint significantly reduced spleen, F (1,31) = 26.89, $p < 0.0001$, and thymus weights, F (1,31) = 348, $p < 0.0001$, in both the infected and non-infected groups. Although adrenal weights were also reduced, this effect failed to reach statistical significance, F (1,31) = 3.84, $p = 0.07$. Also, neither the main effects of infection nor the interactions between infection and restraint were significant, all Fs (1,32) < 2.72, $p > 0.05$.

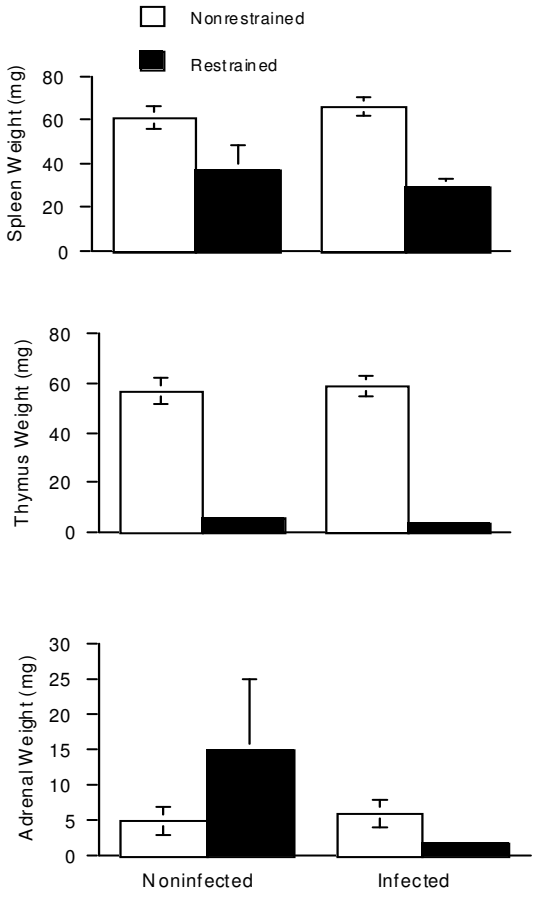

Figure 2-5. Effect of restraint stress on organ weights. The weights of spleen and thymus were considerably decreased after stress, but not affected by infection. Data are expressed as the mean $\pm$ S.E.M.

3.3 Chemokine alterations in the brain following Theiler's virus infection and restraint stress

To determine whether Theiler's virus infection and restraint stress alter the expression of chemokines involved in the recruitment of inflammatory cells, brains were collected at days 2 and 7 p.i. for RNase protection assay (Figure 2-6). As depicted in Figure 2-6, infection tended to increase the levels of several chemokines in the brain at day 7 p.i., whereas restraint stress selectively attenuated infection-related increases in IP-10, RANTES and Ltn. For each time point, a series of ANOVAs were conducted for each chemokine entering infection and restraint as between subject variables.

At day 2 p.i., infection and restraint did not alter chemokine expression, all Fs (1,11) < 3.044, ps > 0.05. In contrast, at day 7 p.i. significant main effects for infection were observed for IP-10, RANTES, Ltn, MCP-1, and TCA-3, wherein infection elevated expression of these cytokines, all Fs (1,31) > 7.85, ps < 0.009. Although eotaxin appeared to be elevated by infection, this difference failed to reach significance, F (1,31) = 1.95, p = 0.17. These findings indicate that Theiler's virus infection results in a selective pattern of chemokine expression and that these chemokines may be involved in the local virus-induced immune response that recruits inflammatory cells into CNS.

Restraint stress appears to decrease the level of infection-related expression of IP-10, RANTES, and Ltn on day 7 p.i.. Supporting this, significant main effects of restraint stress were observed on IP-10, RANTES, and Ltn expression on day 7 p.i., all Fs (1,31) > 3.96, ps < 0.05, but not day 2 p.i., all Fs (1,11) < 3.044, ps > 0.05. Importantly, these main effects were qualified by significant restraint by infection interactions for IP-10, RANTES, and Ltn, all Fs (1,31) > 4.28, ps < 0.05, suggesting that the effect of restraint

stress depended on infection status. Post hoc mean comparisons confirmed that the infection-related increases in these chemokines were decreased by restraint stress, all *p*s < 0.05. Other chemokines were not altered by restraint or infection at day 7 p.i., including MIP-1α, MIP-1β, and MIP-2, all *F*s (1,31) < 1.698, *p*s > 0.05. No other differences were significant. Taken together, these results suggest that restraint stress decreases infection-related increases for IP-10, RANTES, and Ltn expression, which would be expected to decrease the recruitment of inflammatory cells into the CNS.

To characterize the temporal dynamics of the cytokine response in brain, a series of ANOVAs were conducted for each chemokine entering time of sacrifice, infection and restraint as between subject variables. Main effects for time of sacrifice were found for the chemokines IP-10, RANTES, Ltn, MCP-1, TCA-3, and MIP-1β, all *F*s (1,42) > 3.84, *p*s < 0.05. Transcripts of these chemokines were minimal at day 2 p.i., but elevated at day 7 p.i., indicating that the induction of these chemoattractants was enhanced over time. Importantly, significant two-way interactions were observed between infection and time of sacrifice for IP-10, RANTES, and Ltn, all *F*s (1,42) > 4.08, *p*s < 0.05, indicating that the level of virus-induced chemokine expression increased over time. There also appeared to be an interaction between infection and time for MCP-1 and TCA-3, but these interactions failed to reach significance, all *F*s (1,42) > 3.134, *p*s = 0.08. The two-way interactions between time of sacrifice and restraint stress, all *F*s (1,42) < 2.193, *p*s > 0.146, and the three-way interactions between time, infection, and restraint were not significant for any of these chemokines, all *F*s (1,42) < 2.674, *p*s > 0.11.

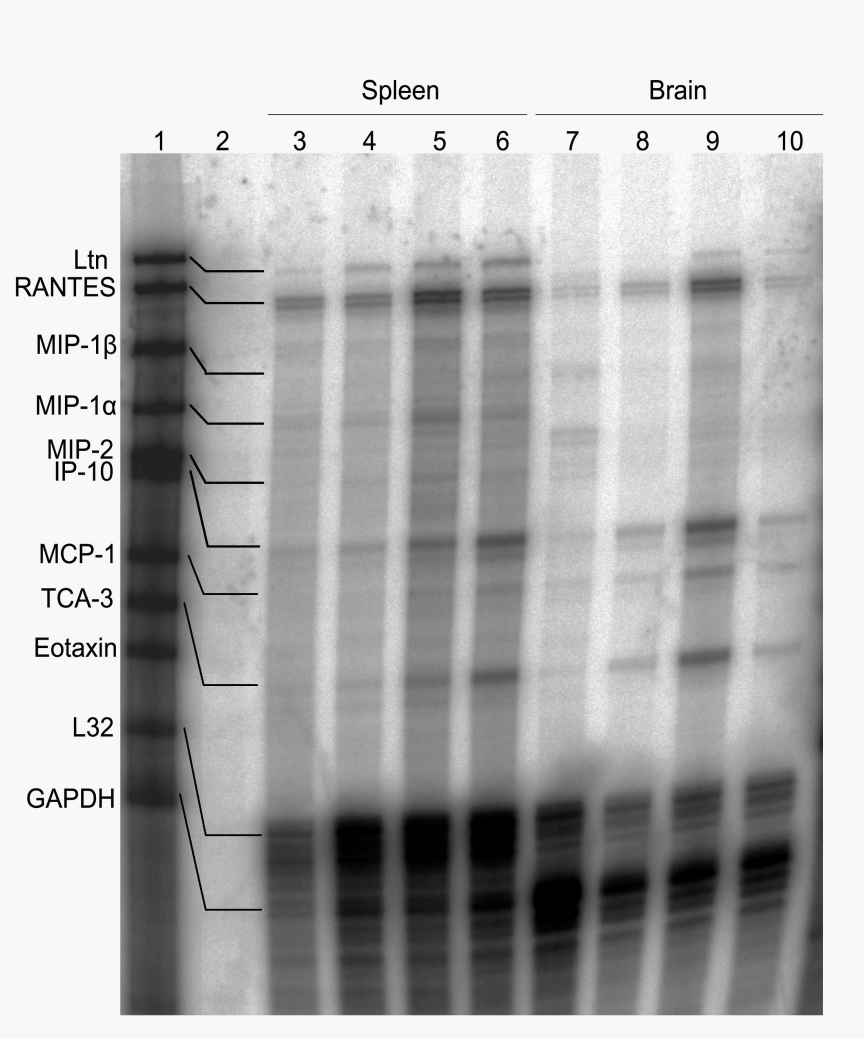

Figure 2-6. Phosphor image of a representative RPA for the chemokine expression in the spleen (lane 3-6) and brain (lane 7-10) following restraint and/or BeAn infection at day 7 post infection. Total RNA was isolated from the brains and spleens of CBA mice at day 7 postinfection. 25µg RNA was hybridized with a ^{32}P-labeled chemokine probe set as described in Materials and Methods. The housekeeping genes L32 and GAPDH were used for the normalization of sample loading and technique error. Lane 1, a chemokine probe; lane 2, the corresponding RNase-protected probes following hybridization with yeast tRNA to testify the assay performance. Lane 3,4,5,6, and lane 7,8,9,10 are the spleen and brain samples respectively collected at day 7 postinfection. NI/NR (Non-infected/Non-restrainedl), NI/R (Non-infected/Restrained), I/NR (Infected/Non-restrained) and I/R (Infected/Restrained).

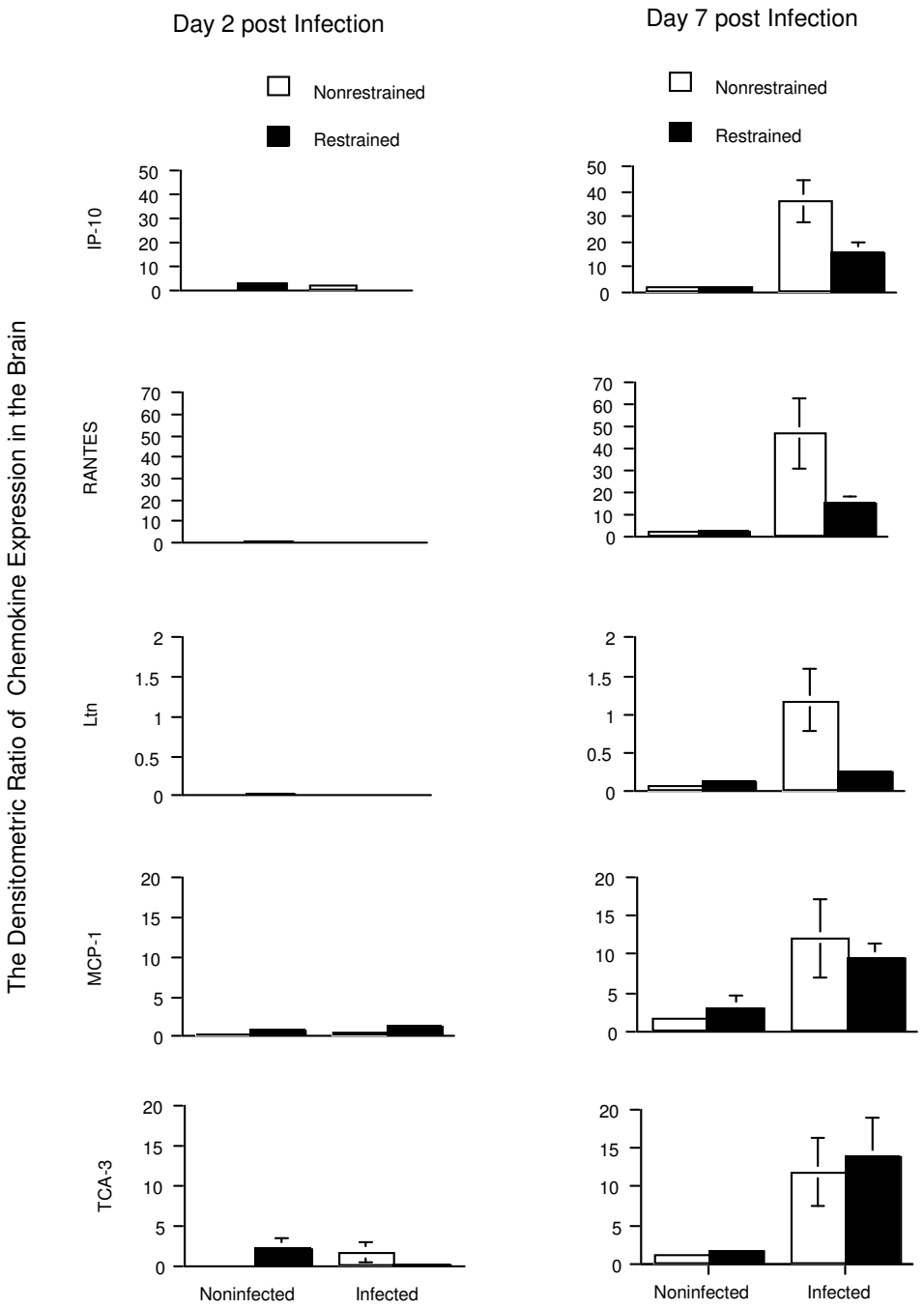

Figure 2-7. The effect of restraint stress on chemokine expression in the brain at days 2 and 7 p.i. during acute TMEV infection. The levels of chemokines were derived from the densitometric ratio of individual chemokines to the housekeeping gene GAPDH and multiplied by 1000. At day 2 p.i., chemokine levels were not changed by stress nor infection. At day 7 p.i., infection increased mRNA expressions of IP-10, RANTES, Ltn, MCP-1 and TCA-3. Restraint stress decreased infection-related increases in the production of IP-10, RANTES, and Ltn at day 7 p.i. as compared to non-restrained mice. The values are expressed as the mean $\pm$ S.E.M.

3.4 Chemokine alterations in the spleen after infection and restraint stress

To explore the effect of stress on the peripheral immune response to TMEV, we examined the expression of chemokines in the spleen at days 2 and 7 p.i. (Figure 2-8). In contrast to the brain, several chemokines were up-regulated in spleen as early as day 2 p.i., and restraint stress attenuated this response. A series of ANOVAs were conducted for each chemokine entering infection and restraint as between subject variables for each time point.

Significant main effects of infection were found at day 2 p.i. for IP-10, Ltn, MCP-1, MIP-1β, and TCA-3, all Fs (1,13) > 5.516, ps < 0.04, indicating that infection alone increased expression of these splenic chemokines. However, MIP-1α, eotaxin, MIP-2, and RANTES expression were not altered by infection at day 2 p.i., all Fs (1,13) < 1.11, ps > 0.05. Because the chemokine response was detected earlier in spleen than in brain, suggests that the amplification of immune response to Theiler's virus infection was generated peripherally. At day 7 p.i., IP-10, Ltn, MIP-1β, TCA-3, RANTES, and MIP-2 were significantly elevated by infection, all Fs (1,33) > 6.175, ps < 0.0182; but, there was no effect of on MCP-1 or TCA-3, all Fs (1,13) < 0.335, ps> 0.05.

Restraint stress reduced levels of IP-10, Ltn, MCP-1, MIP-1β, RANTES, and TCA-3 at day 2 p.i., all Fs (1,13) > 16.804, ps < 0.001, and at day 7 p.i. for RANTES only, F (1,33) = 4.272, p < 0.05. Although restraint appeared to decrease levels of MIP-1β, MCP-1, and MIP-1α at day 7 p.i., these differences were not statistically significant, all Fs (1,33)<2.93, ps > 0.10. These findings were qualified by significant restraint by infection interactions at day 2 p.i. on the expression of IP-10, MIP-1β, MCP-1 and TCA-3, all Fs (1,13) > 6.179, ps < 0.03, but not at day 7 p.i., all Fs (1,33) < 3.131, ps > 0.05,

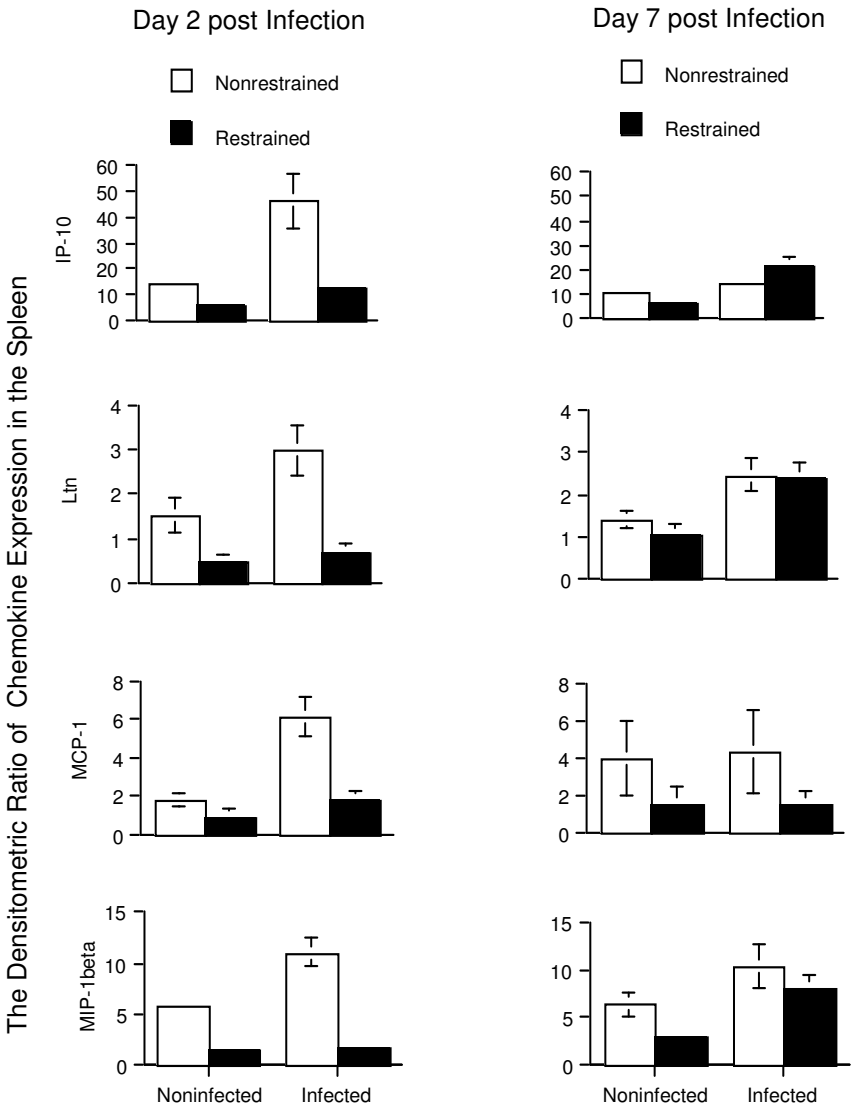

Figure 2-8. The impact of restraint stress on chemokine expression in the spleen at days 2 and 7 p.i. during acute TMEV infection. The levels of chemokines were derived from the densitometric ratio of individual chemokine to the housekeeping gene L32 and multiplied by 1000. At day 2 p.i., infection increased IP-10, Ltn, MCP-1, MIP-1β, and TCA-3. At day 7p.i., IP-10, Ltn, MIP-1β, TCA-3, RANTES, and MIP-2 were elevated by infection, but MCP-1 and TCA-3 subsided at this time point. The expression of virus-induced IP-10 was decreased over time, while MIP-1α and MIP-2 were upregulated over time. Stress significantly suppressed the expression of IP-10, Ltn, MCP-1, MIP-1β, RANTES, and TCA-3 as early as day 2 p.i.; however, this effect was only seen in RANTES expression on day 7 p.i. The values were represented by the mean $\pm$ S.E.M.

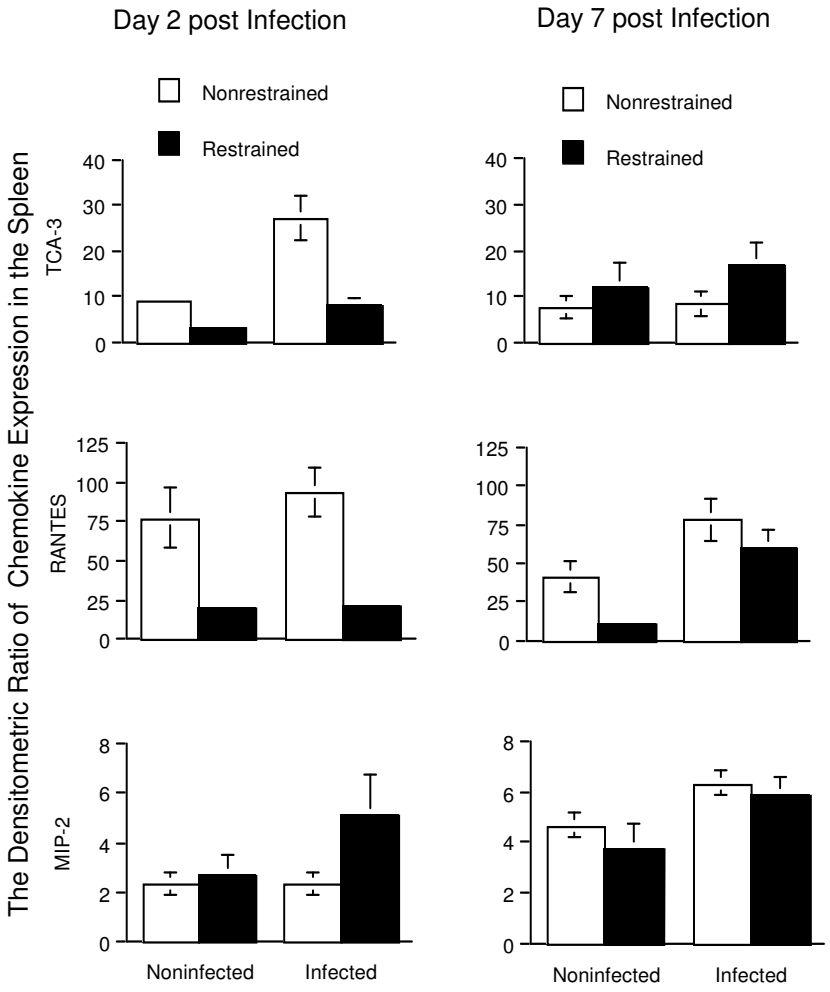

Figure 2-8. continued.

suggesting that the suppressive effects of stress were attenuated over time. Post hoc mean comparisons verified that the infection-related increases in these chemokines were suppressed by restraint at day 2 p.i., all *p*s < 0.05. Similar to the early chemokine response to infection observed in spleen, the immunosuppressive effects of stress on chemokine expression occurred more rapidly in spleen (day 2 p.i.) than in brain (day 7 p.i).

To further explore how the cytokine response in spleen changed over time, a series of ANOVAs were conducted for each chemokine entering time of sacrifice, infection and

restraint as between subject variables. Significant main effects for time of sacrifice were observed for IP-10, MIP-1α, and MIP-2, indicating that IP-10 was down regulated, F (1,46) = 4,637, $p < 0.04$, and MIP-1α and MIP-2 were up regulated over time, all Fs (1,46) > 9.08, ps < 0.0004. Significant interactions between restraint and time of sacrifice were found for IP-10, RANTES, Ltn, and TCA-3, all Fs (1,46) > 4.405, ps <.04.

In general, restraint suppressed the expression of these chemokines at day 2, but restraint either had no effect (IP-10, RANTES, lymphotaxin,) or increased (TCA-3) expression at day 7. Although the interactions between infection and time sacrifice failed to reach significance, all Fs (1,46) > 3.059, ps > 0.09, a significant three-way interaction between infection, restraint, and time was observed for IP-10, F (1,46) = 9.563, $p < 0.003$. This interaction was attributable to a restraint-induced suppression of IP-10 in infected mice at day 2 p.i. followed by a reversal of this effect of restraint at day 7 p.i..

3.5 Histopathology findings

Previously we examined the effect of restraint stress on CNS inflammation in mice infected with TMEV at day 7 p.i. following five nights of RST and two nights without stress (Campbell et al., 2001). In the current experiments, the mice were subjected to restraint stress each night for a total of seven nights.

A mild to moderate focal nonsupprative meningoencephalitis characterized by perivascular cuffing, microgliosis and meningitis occurred in the hippocampus of infected/non-restrained (I/NR) mice (see Figure 2-9C and Table 2-2. meningitis not shown). In contrast, there was a very mild pattern of inflammation, manifested by decrease in cuffings and microgliosis in the hippocampus and meninges of

infected/restrained (I/R) mice (Figure 2-9D), illustrating the marked effect of continuous restraint stress on the severity of encephalitis. Additionally, there was no obvious morphological change of neural cells and no inflammation in the hippocampus of the noninfected/restrained (NI/R) mice (Figure 2-9B).

The pathology changes were also confirmed by a series of ANOVAs performed on perivascular cuffing, microgliosis and meningitis scores. Across all measures, significant main effects were found for infection, all Fs $(1,11) > 11.65$, $p < 0.06$, and restraint, all Fs $(1,11) > 11.65$, $p < 0.06$. More importantly, significant interactions between infection and restraint stress were also observed, all Fs $(1,11) > 6.181$, $p < 0.03$. Post hoc mean comparisons confirmed that restraint stress strikingly reduced the number and severity in perivascular cuffings, microgliosis and meningitis in the infected mice, all ps < 0.003 (Table 2-2).

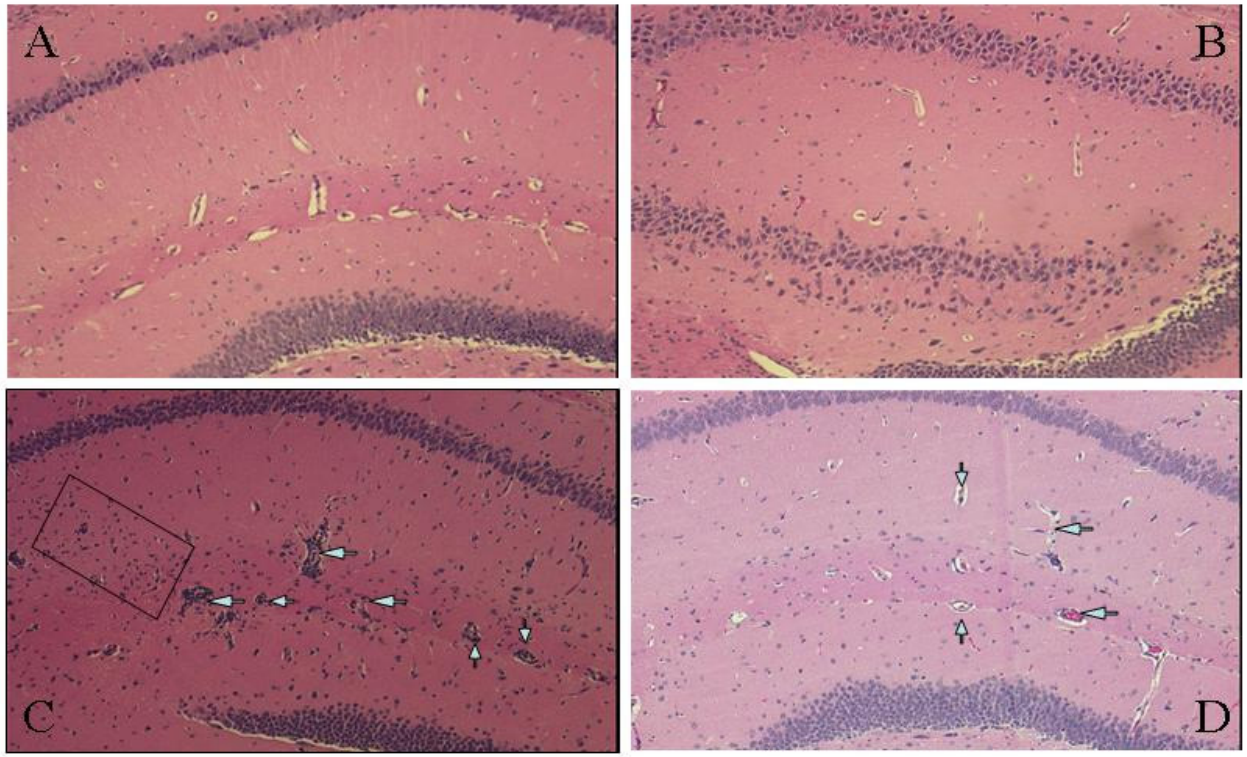

Figure 2-9. H&E stained sections of hippocampus collected at day 7 post infection (50X). (A).Non-infected/Non-restrained. (B)Non-infected/Restrained. (C) Infected/Non-restrained hippocampus. Mild to moderate microgliosis (as shown in the box) and perivascular cuffing (indicated by arrow). (D) Infected/Restrained. Mild perivascular cuffing (indicated by arrow).

Table 2-2 Histological assessment of H&E stained brain section

Features	non-infected/ non-restrained	non-infected/ restrained	infected/ non-restrained	infected/ restrained
Perivascular cuffs	0±0	0±0	7.267 ± 2.800 *	0.320 ± 0.121 *
Meningitis	0±0	0±0	0.047 ± 0.018 *	0.007 ± 0.004 *
Microgliosis	0±0	0±0	0.427 ± 0.137 *	0.035 ± 0.035 *

Histological score was evaluated as described in the methods and materials. The scores are represented by mean ± S.E.M. Prominent pathological changes of perivascular cuffs, meningitis and microgliosis were observed in the infected/non-restrained barins, and these alterations were markedly reversed by stress. * indicates $p < 0.001$.

4. Discussion

The current study represents part of a series of investigations into the effects of restraint stress on the neuropathogenesis of Theiler's virus infection in mice. We showed the profound effect of restraint stress as evidenced by worsened clinical signs, decreased body weight and reduced spontaneous activity during the acute infection. We have previously demonstrated the detrimental effects of restraint stress on this murine infection and have proposed that the mechanism involved is primarily mediated by a stress-induced immunosuppression resulting in a decreased inflammation of the CNS and consequently an impaired viral clearance (Campbell et al., 2001). One of the possible mechanisms for decreased CNS inflammation is reduced chemokine expression during the early stages of Theiler's virus infection, as chemokine expression in the CNS has been demonstrated to be involved in the recruitment of immune cells (Theil et al., 2000; Hoffman et al., 1999; Murray et al., 2000). Therefore, the current study investigated the effects of restraint stress on chemokine expression in mice infected with Theiler's virus.

We will firstly focus on the effect of viral infection on mRNA chemokine levels in the brain (Table 2-3 and Table 2-4). In this study, intracerebral inoculation of the BeAn strain of Theiler's virus did not induce significant chemokine expression in the brain at day 2 p.i., but did selectively induce mRNA elevation of CXC chemokine: IP-10/CXCL10; C chemokine: Ltn/XCL1; and three CC chemokines: TCA-3/CCL1, MCP-1/CCL2 and RANTES/CCL5 in the brain of CBA mice at day 7 p.i.. A similar profile of chemokine increases during the early phase of TMEV infection has previously been reported (Murray et al., 2000; Theil et al., 2000) in other strains of mice.

Table 2-3 Summary of the chemokine changes in the brain and spleen at day 2 post infection

Day2 p.i.	BRAIN			SPLEEN		
Chemokines	Infection	Restraint	Infection by Restraint	Infection	Restraint	Infection by Restraint
Ltn				(+)	(---)	
IP-10				(+++)	(---)	(-)
RANTES					(---)	
MCP-1				(+++)	(---)	(-)
MIP-1alpha						
MIP-1 beta				(++)	(---)	(---)
MIP-2						
TCA-3				(+++)	(---)	(-)
Eotaxin						

(+/-): $p < 0.05$; (++/--): $p < 0.01$; (+++/---): $p < 0.001$; blank: unchanged

Table 2-4 Summary of the chemokine changes in the brain and spleen at day 7 post infection

Day7 p.i.	BRAIN			SPLEEN		
Chemokines	Infection	Restraint	Infection by Restraint	Infection	Restraint	Infection by Restraint
Ltn	(++)	(-)	(-)	(+++)		
IP-10	(+++)	(-)	(-)	(+)		
RANTES	(+++)	(-)	(-)	(+++)	(-)	
MCP-1	(+++)					
MIP-1alpha						
MIP-1 beta				(+)		
MIP-2				(+)		
TCA-3	(+++)					
Eotaxin						

(+/-): $p < 0.05$; (++/--): $p < 0.01$; (+++/---): $p < 0.001$; *blank: unchanged*

There are various sources of chemokines for instance: IP-10 is produced by astrocytes, oligodendrocytes, microglia, and endothelial cells and is a chemoattractant for activated T cells and monocytes (Salmaggi et al., 2002; Palma et al., 2001). IP-10 can also facilitate T cell adhesion to endothelial cells (Taub et al., 1993) and participate in T cell priming (Dufour et al., 2002). The cellular sources of RANTES include astrocytes, oligodendrocytes, microglia, and activated memory T cells (Hvas et al., 1997; Palma et al., 2001) and RANTES is also involved in the recruitment of memory T cells and monocytes. In previous TMEV studies, it was reported that expression of CNS IP-10 and RANTES displayed similar kinetics, in which both chemokines were increased during the encephalitis phase and remained at a relatively high level throughout the disease (Hoffman et al., 1999; Murray et al., 2000; Theil et al., 2000). The parallel expressions of IP-10 and RANTES were seen in both acute and chronic stage of experimental allergic encephalomyelitis (EAE) model (reviewed in Elhofy et al., 2002), mouse hepatitis virus (MHV)-induced demyelinating disease (reviewed in Glass et al., 2002) and was also detected in the CSF of MS patients (Sorensen et al., 1999). These observations suggest that the elevation in the T cell chemoattractants, IP-10, and RANTES, coincide with the immune response to the viral infection and also with the development of demyelination. Furthermore, it indicates that these chemokines may be involved in the accumulation of T cells in the CNS and in maintaining the inflammatory response.

MCP-1 is induced by both Theiler's virus and MHV infection during the acute disease, and is also detected in the CNS of rats during acute EAE. During the remission of EAE, MCP-1 became undetectable and then resurged as the disease progressed into the relapsing phase, demonstrating that its level and kinetics correlated with the disease

development (see reviews by Karpus and Ranoshoff, 1998). Macrophages, astrocytes, and T cells, which all possess the main MCP-1 receptor, CCR2, may accumulate in the CNS following attraction by MCP-1 in EAE (Jee et al., 2002). Conversely, in vitro, anti-MCP-1 remarkably inhibited the migration rate of lymphocytes isolated from MS patients by 60% either with neutralizing antibody or translational arrest of MCP-1 mRNA. Inhibition of MCP-1 can also reduce monocyte adhesion (Maus et al., 2002), indicating that inflammatory cell migration through brain endothelium is MCP-1 dependent to some extent (Prat et al., 2002). In addition, MCP-1 can also enhance NK cell migration and NK cell-mediated cytolysis (Allavena et al., 1994). NK cells are known to be important in early viral clearance of Theiler's virus (Paya et al.,1989).

TCA-3 was also elevated in the brains of mice infected with Theiler's virus as determined by RPA. TCA-3 expression was detected in EAE 1-2 days prior to the onset of clinical signs and was produced by activated encephalitogenic T cells (Godiska et al., 1995). Interestingly, polymorphisms have been described in the chemokine genes TCA-3 (*Scya1*), MCP-1 (*Scya2*) and MCP-5 (*Scya12*), which have also been suggested to be candidates for a locus (*eae7*) controlling susceptibility to monophasic remitting/nonrelapsing EAE in SJL mice, but not in EAE-resistant strains B10.S/DvTe and Balb/cJ mice (Teuscher et al., 1999). The increased expression of TCA-3 in the current study might imply that the virus-induced TCA-3 plays a role in the susceptibility of CBA mice to Theiler's virus infection and the later development of demyelination.

Ltn mRNA levels were also increased as a result of Theiler's virus infection. Ltn is a novel γ-chemokine, produced by activated T cells and NK cells, and is also a specific chemoattractant for T cells and NK cells (Giancarlo, et al., 1996; Hedrick et al., 1997;

Kelner et al., 1994). Moreover, when mice were infected with an adenovirus vector over expressing Ltn, the kinetics of Ltn production was closely correlated with the number of cellular infiltrates (CD4+, CD8+ and NK cells) in the lungs (Emtage et al., 2002).

MIP-2, an important mouse neutrophil chemoattactant (Wolpe et al., 1989), was not significantly changed in the brain after infection in our study, which may account for the lack of neutrophils in the inflammatory infiltrate of CNS.

Taken together, the increased expression of multiple chemokines induced by Theiler's virus infection may account for the recruitment of inflammatory cells, observed in the meninges and hippocampus of the infected brain in this study. We speculate that the inflammatory cells consist of T cells, NK cells, macrophages and also resident microglia. Our results demonstrated that the time of sacrifice was correlated with the expression of virus-induced chemokines, which include Ltn, IP-10 and RANTES. The up-regulation of these chemokines implied that recruitment of immune cells was increased over time. The source of the elevated chemokines is unknown, but could either be CNS resident glia or peripheral immune cells.

We found an altered chemokine pattern in the spleen of virus-infected mice at day 2 p.i., but no virus-induced chemokine expression in the brain at this same time point; the spectrum of chemokine expression in the spleen at day 2 p.i. was similar to that in the brain at day 7 p.i., which included increased Ltn, IP-10, TCA-3, MCP-1, MIP-1β, but not RANTES in the spleen. The similarity of the chemokine spectrum and the delayed reaction in the CNS again suggest that the immune response to Theiler's virus is initiated in the periphery.

Secondly, considering the effects of restraint stress on chemokine expression in the brain (Table 2-3 and Table 2-4), we detected significantly decreased levels of the virus-induced upregulation of brain IP-10, RANTES, and Ltn in the stressed mice. This may account for the reduced degree of inflammation in the brain (e.g. meninges and hippocampus) of infected/restrained animals, which would be compatible with a suppressed chemoattractive influence. Restraint also affected the induction of chemokines in the spleen as shown in our results. A broader spectrum of chemokines than seen in the brain, was considerably decreased by stress, which included IP-10, Ltn, RANTES, MIP-1β, MCP-1 and TCA-3 at day 2 p.i., and RANTES only at day 7 p.i.. Thus, stress profoundly impacts the peripheral immune system, resulting in the inhibition or suppression of the systemic immune response to pathogens. The alterations of chemokine profile over time, as shown by statistical analysis, revealed that the levels and the variety of chemokine expression were notably reduced from day 2 p.i. to day 7 p.i., which suggested the suppressive effects of restraint were attenuated over time.

Exposure to chronic stress has been shown to compromise the immune response to viral infection. It is well known that restraint stress induces an elevation of serum glucocorticoid (GC), which alters inflammatory cell trafficking to draining lymph nodes and inflamed sites; and an increase of catecholamines produced by sympathetic nervous system (SNS), which can suppress the activation of virus-specific T cells (Bonneau et al., 1991 and 1993; Dobbs et al., 1993; Feng et al., 1991; Hermann et al., 1993, 1994a and b; Kusnecov et al., 1992; Padgett et al., 1998; Sheridan et al., 1998). GC is a stress hormone that confers multiple functions physiologically and pathologically. For example, cytokine synthesis and its actions are affected by GC following stress, which has been attributed to

altered immune cell redistribution and migration (e.g. Chung et al., 1986; Fingerle-Rowson et al., 2003; Perretti, et al., 1994; Steer et al., 1998). This suppressive action is caused by inhibition of transcription, translation, secretion, and destabilization of cytokine mRNA (e.g. IL-1 and TNF-α) (reviewed in Sapolsky et al., 2000), which alter leukocyte migration (Perretti, et al., 1994; Steer et al., 1998). Generally, the extravasation and chemotaxis of circulating leucocytes into tissues are governed by vascular adhesion molecules, such as ICAM, VCAM, selectin, and various chemokines, that enhance the movement of cells to inflammatory sites. Tailor et al. (1999) found that dexamethasone could inhibit the secretion of IL-1-induced ICAM-1 and chemokine KC, and consequently reduce leukocyte adhesion. GC's have also been shown to inhibit RANTES mRNA in T cells and lung endothelial cells (Kwon et al., 1995; Wingett et al., 1996). Mizobe et al. (1997) documented that the down-regulation of MCP-1 and a reduced migration of macrophage and granulocytes to injured sites following acute restraint stress was mediated by GC.

The underlying mechanism of GC's interference with chemokine production is not fully understood. It is thought that the immunosuppressive actions of GC's are primarily mediated through the binding of GC to glucocorticoid receptors (GR's), forming a GR complex, which translocates to the nucleus, targeting the inflammatory mediator genes that regulates their expression (reviewed in Sapolsky et al., 2000; Marchetti et al., 2001) or affects NF-κ B activation (Ohtsuka et al., 1996). Smith et al. (1997) identified several chemokine genes (MCP-1, 3, IP-10) that were attenuated by GC following exposure to LPS stimuli, supporting the hypothesis that chemokine expression may be regulated by GC's.

In addition to glucocorticoid, the products of the SNS have been considered to contribute to cell trafficking during restraint stress. For example, reduced cellularity of inflamed tissue can be restored in mice pretreated with 6-hydroxydopamine (6-OHDA), a neurotoxin which is readily taken up by adrenergic nerve terminals that blocks the binding of catecholamine (Hermann et al., 1994), illustrating the inhibitory role of catecholamine in inflammatory cell migration. The cross talk between the nervous and immune systems is based on the innervation of mononuclear cells within lymphoid organs by extensive efferent nerve endings of SNS. T cells and B cells carry β-adrenergic receptors and macrophages bear α and β adrenergic receptors. Furthermore, it has been shown that MIP-1α generated by monocytes can be inhibited by adrenaline (Li et al., 2003). Therefore, SNS-mediated down-regulation of chemokines following stress may account for the results obtained in the spleens in the current study. The spleen is one of the major lymphoid organs for T and B cells activation while the thymus is the location for T cell maturation. The significant weight loss of the spleen, as well as the prominent thymic atrophy observed in the current study could be explained by diminished cellularity that resulted from increased plasma glucocorticoid, which has direct toxic effect on immune cells, and/or from apoptosis (Tarcic et al., 1998; Yin et al., 2000), thus eventually leading to reduced production of chemokines.

In conclusion, restraint stress applied to mice infected with Theiler's virus resulted in decreased cellularity in the CNS. We hypothesize that the decreased CNS inflammation occurs as a result of reduced expression of the chemokines Ltn, IP-10, RANTES and MCP-1, which are chemoattractants for $CD4^+$, $CD8^+$ T cells, macrophages and NK cells. It should be noted that the current study focused on the effect of stress and infection on

the expression of chemokines at transcriptional level and did not include determination of protein levels and their correlations with mRNA. Therefore, the alterations of certain chemokine transcripts may not be consistent with protein changes. Secondly, the specific functions of these chemokines in TVID and the phenotype of the cells within the lesions require further investigation. However, this study delineated possible mechanisms by which stress impacts the local and systemic immune response to a neurotropic viral infection. In this model, stress has profound effects on the early events during infection with Theiler's virus which has important implications in our understanding of how stress may play a role in the establishment of autoimmune disease.

III THE EFFECT OF RESTRAINT STRESS ON CYTOKINE EXPRESSION IN THE CNS AND SPLEEN DURING THE ACUTE TMEV INFECTION

1. Introduction

The etiology of multiple sclerosis (MS) remains uncertain and multiple factors, such as genetic predisposition and external environmental factors, such as infectious pathogens, psychological stressors and climates, have been reported to be linked to the pathogenesis of MS. Infectious agents, such as viruses have been associated with neurological disorder (Gilden, 2005). Theiler's murine encephalomyelitis virus-induced demyelination (TVID) has served as an animal model to investigate infectious mechanisms mediating central nervous system (CNS) demyelination in human MS. Intracerebral inoculation of Theiler's virus, a natural murine pathogen, produces a biphasic CNS disease in susceptible mice. The acute phase of disease involves a mild encephalomyelitis with predominant neuronal infection, whereas the chronic phase is characterized by inflammatory demyelination in white matter with glial and microglial infection (Lipton et al., 1975). Unlike the resistant strains of mice, which are able to clear the virus during acute infection and do not develop demyelination, the susceptible strains of mice fail to clear virus and a persistent CNS infection is established that is essential for the induction of demyelination in the spinal cord (Lipton et al., 1984; Rodriguez et al., 1983). Although it is evident that the susceptibility to Theiler's virus infection and demyelination is genetically regulated (Brahic and Bureau, 1998), the immune response also plays a critical role in determining the viral load and the degree of

viral persistence, thus effecting the progression and severity of demyelinating disease (Aubagnac et al., 1999; Rodriguez et al., 1996).

The acute encephalomyelitis is primarily mediated by both innate and adaptive antiviral immunity via infiltrating $CD4^+$, $CD8^+$ T cells (reviewed in Oleszak et al., 2004) and NK cells (Paya et al., 1989), while the adaptive immunity contributes to the late demyelination, by delayed-type hypersensitivity (DTH) responses to viral antigens via $CD4^+$ T cells (Borrow et al., 1993; Gerety et al., 1994).

In response to Theiler's virus infection, immune cells produce a variety of inflammatory mediators, such as cytokines, chemokines and enzymes. Cytokines play a crucial role in the regulation of the immune response in clearing the virus from the CNS and mediating demyelination. It has been shown that a panel of dominant pro-inflammatory (Th1) cytokines, such as interferon-γ (IFN-γ), tumor necrosis factor (TNF)-α, interleukin (IL)-6, interleukin (IL)-12, etc, was induced during acute encephalomyelitis in all strains of mice, but at a higher level in susceptible mice than resistant mice (Chang et al., 2000; Sato et al., 1997; Theil et al., 2000). Some Th1 cytokines, for example, IFN-γ, exhibit antiviral activity as observed in knockout mice (Rodriguez et al., 2003). In addition, Th1 cytokines may further activate and regulate other immune cells, such as antigen presenting cells and microglia, thus promoting protection against viral infection (reviewed by Oleszak et al., 2004).

After the first two weeks of infection, Th1 cytokine levels decline in resistant mice, which correlates with the clearance of virus from the CNS; whereas in susceptible strains of mice, Th1 cytokines remain at relatively high levels and are partly responsible for the progression of demyelination (Begolka et al., 1998; Chang et al., 2000; Murray et al.,

2002; Sato et al., 1997; Trottier et al., 2004). It has been shown that anti-inflammatory (Th2) cytokines may alleviate demyelination in TVID (Drescher et al., 2000), and this is also seen in the remission of experimental allergic encephalomyelitis (Begolka et al., 1998).

The specific Th1/Th2 cytokine paradigm found in animal models has not always been seen in MS patients and the correlation between the Th1/Th2 cytokine paradigm and clinical variables may not always be consistent. The fluctuations of MS seem to be related with the upregulation of both Th1 and Th2 cytokines (Balashov et al., 2000; Link, 1998). In contrast, the opposite observation was also reported that a reduction in Th1 and Th2 T cell phenotypes in active disease (Matsui et al., 2004). These results suggest dysregulation of Th1/Th2 cytokine paradigm in MS. However, the association of Th1/Th2 balance with clinical manifestations of MS is supported by the effectiveness of several therapeutic agents, such as ibudilast and glatiramer acetate, which can ameliorate the disease by diminishing the Th1 response and generating Th2 dominance (Feng et al., 2004; Vieira et al., 2003). The mechanism of triggering or exacerbating MS is unknown, but it has been proposed that stressful life events may contribute to, or have an effect on the flare-ups of MS (Mohr et al., 2004).

The stress response involves a complex interaction between the neuroendocrine and the immune systems, resulting in alterations in both innate and adaptive immunity to various pathogens and stimuli, thus enhancing the risk or vulnerability to inflammatory and autoimmune diseases, as well as infectious disease. The mechanisms by which stress modulates the pathogenesis or progression of these diseases are not fully understood. Two major pathways, the hypothalamic-pituitary-adrenal (HPA) axis and the

systemic/adrenomedullary sympathetic nervous system (SNS) are activated in response to stress, resulting in the production of glucocorticoids (GC) and catecholamines (CA) from the two systems. GC, CA as well as other stress hormones, opioids, growth hormones and prolactin, impact the function of immune system (Pruett, 2003). Among these stress hormones, the role of glucocorticoids in the immune response has been extensively studied. It has been widely used as an immunosuppressor or anti-inflammatory agent in the therapy of many autoimmune diseases, including MS.

On the other hand, the effectors of immune cells, such as cytokines, secreted in response to stress stimulation, can influence the neuroendocrine system via cytokine receptors, thus providing links between the two systems (reviewed in Haddad et al., 2002). Therefore, cytokines appear to be pivotal factors in the pathogenesis of MS and the stress response.

We employed a restraint stress model, to mimic both the psychological and physiological effects of stress in the Theiler's virus model in order to investigate the underlying mechanism by which stress affects MS. Previous studies from our laboratory have shown that stress increased serum corticosterone (Campbell et al., 2001), decreased NK cell activity (Welsh et al., 2004), reduced chemokine expression and CNS inflammation (Mi, et al., 2004) during the acute infection. In this study, we further examine the cytokine expression following restraint stress and Theiler's virus infection and the stress-induced Th/Th2 cytokine shift. These studies should enable us to gain some insight of the role of environmental factors, stressor and virus, on the pathogenesis of MS.

2. Materials and methods

2.1 Subjects and grouping

3-week-old male CBA mice were obtained from Halan Laboratories (Indianapolis, IN) and were maintained on a 12 h light/dark cycle. The animals were counterbalanced by weight and then were allowed to habituate for one week before the initiation of the any experimental procedures.

The mice and were randomly assigned into one of the following four groups using an A 2 (infected vs. non-infected) by 2 (restraint vs. non-restraint) by 2 (2 days of infection vs. 7 days of infection) factorial design: (1) Non-infected/Non-restrained (NI/NR) mice remained undisturbed in their home cages; (2) Non-infected/Restrained (NI/R): each mouse was subjected to restraint stress for 3 consecutive nights (for the mice terminated at day 2 p.i.) or 8 consecutive nights (for the mice terminated at day 7 p.i.) in their home cages; (3) Infected/Non-restrained (I/NR): mice were inoculated intracranially with BeAn virus; or (4) Infected/Restrained (I/R): each mouse was subjected to one-night of restraint stress one day prior to viral inoculation and then was subjected to subsequent stress sessions of 2 or 7 nights (for the mice sacrificed at day 2 p.i. and day 7 p.i., respectively), as described in Mi et al. (2004).

2.2 Restraint stress paradigm

Restraint was applied by placing the mice into well-ventilated restraining tubes (2.7-cm internal diameter and 14-cm length) for 12 h (from 9pm-9am) each for 3 or 8 consecutive nights (Campbell et al., 2001; Mi et al., 2004; Sheridan et al., 1991). The tubes were drilled with small holes to allow adequate ventilation. Restraint stress was initiated 1 day prior to infection and continued for 2 or 7 subsequent sessions.

2.3 Virus and viral inoculation

The BeAn strain of Theiler's virus was obtained from Dr. H. L. Lipton, Department of Neurology, University of Chicago, Evanston, IL and was propagated and amplified in L2 cells as previously described (Welsh et al., 1987).

Following isoflurane anesthesia, $5x10^4$ plaque forming unit (PFU) of the BeAn strain of Theiler's virus in a 20µl volume was inoculated into the right cerebral hemispheres of the mice in the infected groups, or a 20µl of sterile phosphate-buffered saline (PBS) was infected into the non-infected mice.

2.4 Termination of mice

Mice were sacrificed by the i.p. injection of pentobarbital at either day 2 p.i. or day 7 p.i.. Mice were perfused with diethyl pyrocarbonate (DEPC, Sigma)-treated PBS into the right ventricle of the heart. The brains and spleens were aseptically removed, weighed, then were flash frozen in liquid nitrogen and stored at -80^{o}C until used.

2.5 Ribonuclease protection assay (RPA)

Total RNA was extracted from the brain and spleen using TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Two Multi-Probe Template Sets, mCK-2b (IL-12p35, IL-12p40, IL-10, IL-1α, IL-1β, IL-1 Receptor antagonist (IL-1Ra), IL-18/ interferon-gamma-inducing-factor (IGIF), IL-6, IFN-γ, migration inhibitory factor (MIF), and two housekeeping genes L32 (ribosome RNA) and GAPDH (glyceraldehydes-3-phosphate dehydrogenase) and mCK-3b (TNF-β, lymphotoxin-β (LT-beta), IL-6, IFN-γ, IFN-β, transforming growth factor beta 1, 2, 3 (TGF-β1, 2, 3) and

housekeeping genes, L32 and GAPDH) (BD PharMingen, San Diego, CA) were used to examine cytokine expression in the tissues. The probe was labeled with ^{32}P –UTP (800 Ci/mmol, 10μCi/μl, PerkinElmer Life Sciences, Inc., Boston, MA). RPA were conducted using RPA III and MAXIscript kit (Ambion, Austin, TX), according to the manufacturer's instructions.

Briefly, 25μg RNA was hybridized overnight (16 h) with labeled probe and the reactions were subsequently digested with RNase A/T1 Mix, followed by precipitation with Inactivation/Precipitation III solution (Ambion, Austin, TX). The protected RNA-probe complexes were resolved on 5% acrylamide/8M urea/1xTBE gel. Then the gel was dried for 2 h on a gel drier (Gel Drier, Model 583, Bio-Rad), and exposed to a Fujifilm Phosphor Imaging Plate in BAS cassette 2325 (Fuji Medical Systems USA, Inc.) for 14-16 h.

The plate was scanned with a Fujifilm Bio-imaging Analyzer BAS-1800II (Kanagawa, Japan) and the photo-stimulated luminescence (PSL) was analyzed with an ImageGauge software (Version 3.12, Science lab, Fuji Photo Film Co.Ltd). The densitometry of each band was represented by the PSL reading and then normalized with a housekeeping gene (L32 or GAPDH).

2.6 Western blot analysis

While isolating the RNA from the aqueous phase of homogenates using TRIzol, the remaining organic phase was used for further protein isolation as described in the manufacturer's instructions (Invitrogen, Carlsbad, CA). Western blot analyses for TNF-α were conducted in 4-20% Tris-HCl Ready Gels (BioRad, Laboratories, Hercules, CA).

25 μg of protein was loaded into each lane for western blotting. Following electrophoresis, proteins were transferred to a nitrocellulose membrane (Hybond-C super, Amersham, Illinois, USA) on a Trans-Blot SD Semi-dry Transfer Cell and the non-specific binding sites were blocked with 5% Non-fat dry milk in Tween-Tris Buffered Saline (TTBS, 0.1% Tween-20 in 100 mM Tris-CL [pH 7.5], 0.9% NaCl). The membrane was then incubated for 2 h at room temperature with rabbit anti-mouse TNF-α polyclonal antibody (CHEMICON International, Inc.) 0.15 μg/mL in TTBS or rabbit anti-GAPDH monoclonal antibody (Advanced ImmunoChemical Inc, Long Beach, CA, 1:300 in TTBS) for loading control. After three washes with TTBS, the membrane was incubated for 30 min at room temperature with biotinalated donkey anti-rabbit IgG secondary antibody (CHEMICON International, Inc., 1:10000 dilution in TTBS) followed by three TTBS washes. Next, the membrane was incubated with streptavidin-horseradish peroxidase conjugate (Amersham Biosciences, UK, 1:1000 in TTBS). The protein-antibody compounds were revealed with a SuperSignal West Pico Chemiluminescent Substrate kit (PIERCE, Rockford, IL) after four washes with TTBS before exposing to X-OMAT film (Kodak, Rochester, NY). Molecular weights were determined using MultiMark (Invitrogen, Carlsbad, CA). Western blots were scanned for quantification with Image J 1.33U (NIH, USA). The expression of TNF-α was normalized with GAPDH.

2.7 Plaque assay

To correlate the cytokine expression with the viral load in the brains of infected mice, the brains of some of the infected animals were dissected and cut longitudinally along the brain, the left and right hemispheres were alternatively collected for either RPA or viral

titration by a plaque assay. Briefly, the brain was aseptically removed, weighed, and homogenized in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen Carlsbad, CA). Cellular debris was removed by centrifugation and the titer of virus in the supernatant was determined by standard plaque assays on L2 cells.

2.8 Real-time RT-PCR

We analyzed cytokine expression in a subset of the RNA samples by real-time RT-PCR. Total RNA (1μg) was reverse-transcribed into cDNA using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA) according to manufacturers instructions using oligo(dT) or random hexamer primers. Real-time RT-PCR was performed with a GeneAmp 5700 Sequence Detection System (Perkin-Elmer, Norwalk, CT) and SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). Reactions were performed in MicroAmp Optical 96-Well Plates (Applied Biosystems, Foster City, CA) in a volume of 25μL. Data was analyzed using GeneAmp 5700 SDS Software (Perkin-Elmer, Norwalk, CT). For each sample, the cytokine cycle threshold (Ct) values were normalized against β-actin Ct values. Amplification specificity was determined using dissociation curve analysis and, in some cases, by direct sequencing of the PCR product. The following primers were used: 5'β-actin: GCA ACG AGC GGT TCC G; 3'β-actin: TCC AGC CTT CCT TCT TGG G; 5'IL-1β: AGG CAG GCA GTA TCA CTC ATT GT; 3'IL-1β: GGA AGG TCC ACG GGA AAG AC; 5'IL-6: CCA GAA ACC GCT ATG AAG TTC CT; 3'IL-6: CAC CAG CAT CAG TCC CAA GA; 5' IL-10: CAG CCG GGA AGA CAA TAA CTG; 3'IL-10: CCG CAG CTC TAG GAG CAT GT; 5'IL-12p40: AGA CCC TGC CCA TTG AAC TG; 3' IL-12p40: GAA GCT GGT GCT GTA GTT CTC ATA TT;

5'IFN-γ: CAG CAA CAG CAA GGC GAA A; 3'IFN-γ: CTG GAC CTG TGG GTT GTT GAC; 5'LT-β: GAG AGG GTC TAC GTT AAC ATC AGT CA; 3' LT-β: CGC CCC GAA GAA GGT CTT; 5'TNF-α: TGA TCC GCG ACG TGG AA; 3'TNF-α: CCG CCT GGA GTT CTG GAA.

2.9 Statistical analysis

Two-way ANOVA was conducted on the data for the two variables restraint and infection. Duncan's multiple range tests and means comparisons were used for post hoc analyses. A p value of 0.05 or less was considered significant in all cases.

3. Results

3.1 Cytokine expression in the brain following Theiler's virus infection and restraint stress

To assess the potential effect of restraint stress on the immune response in the CNS following Theiler's virus infection, brains were collected at day 2 and 7 p.i. and the cytokine levels were determined by RNase protection assay (Figure 3-1). The main effect of infection, restraint and the interaction between the two variables for each cytokine were conducted by a series ANOVA at the time points of day 2 and 7 p.i.

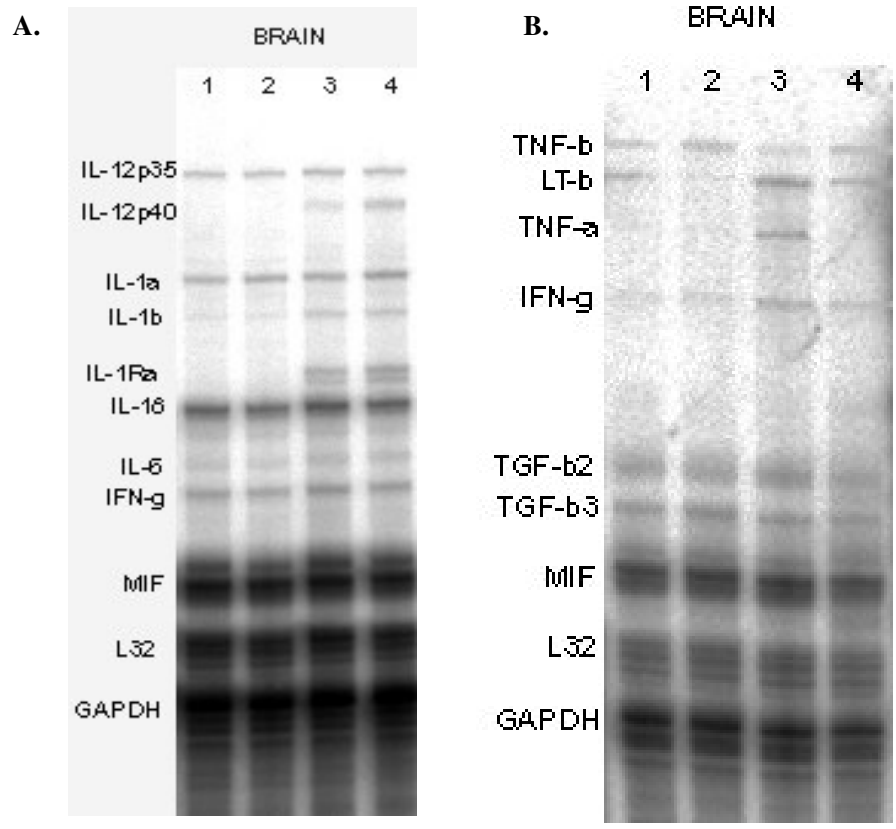

Figure 3-1. Phosphor image of a representative RPA for cytokine expression in the brain following restraint or/and BeAn infection at day 7 post infection using (A) mCK-2b and (B) mCK-3b kits. Total RNA was isolated from the brains and spleens of CBA mice at day 7 p.i. 25μg RNA was hybridized with a ^{32}P-labeled chemokine probe set as described in Materials and methods. The housekeeping genes GAPDH was used for the normalization of sample loading and technique error. Lane 1,2,3,4 represents the brain samples of NI/NR (Non-infected / Non-restrained), NI/R (Non-infected / Restrained), I/NR (Infected / Non-restrained) and I/R (Infected / Restrained), respectively, collected at day 7 post infection.

As summarized in Table 3-1, at day 2 p.i., infection induced a cytokine elevation of LT-β, TNF-α, IFN-γ, IFN-β and IL-6 all *F*s (1,27) > 4.201, *p*s < 0.05. IL-12p40 and MIF were also expressed at a higher level in the brains of infected/non-restrained mice than the normal controls, as confirmed by post hoc mean comparisons, all *p*s < 0.05. At day 7 p.i., a main effect of infection was observed for LT-β, TNF-α, IFN-γ, IFN-β, IL-1β, IL-6 and IL-12p40, all *F*s (1,58) > 3.993, *p*s < 0.05 (Table 3-1).

A panel of fewer cytokines appeared to be affected by restraint stress than infection, wherein restraint increased TNF-β, but decreased LT-β in the brain at day 2 and 7 p.i., all *F*s (1,28) > 6.196, *p*s < 0.02 for day 2 p.i. and all *F*s (1,58) > 6.913, *p*s < 0.01 for day 7 p.i. (Table 3-1).

As depicted in Figure 3-2 and Table 3-1, an interaction between infection and restraint was observed in cytokine expression, wherein restraint significantly decreased the infection-induced IFN-γ at day 2 p.i. [F (1,28) =4.664, p = 0.04] and LT-β at day 7 p.i. [F (1,58) = 8.978, p = 0.004]. Post hoc mean comparison also showed a lower cytokine production in infected/restrained than infected/non-restrained animals in the cytokines LT-β and IL-12p40 at day 2 p.i. and cytokines IFN-γ and IL-6 at day 7 p.i., all *p*s < 0.05, suggesting a suppressive effect on pro-inflammatory cytokine production by stress. Interestingly, INF-β was detected at a higher level in the I/R mice than I/NR mice at day 7 p.i., p < 0.01.

Table 3-1 Summary for alterations in cytokine expression in the brain following TMEV infection and restraint stress

BRAIN	Day 2 post infection			Day 7 post infection		
Cytokines	**INF**	**RS**	**INF and RS**	**INF**	**RS**	**INF and RS**
TNF-α	(+)			(+)		
TNF-β		(+)			(+)	
LT-β	(+)	(-)	(-)	(+)	(-)	(-)
IFN-γ	(+)		(-)	(+)		(-)
IFN-β	(+)			(+)		(+)
IL-1β				(+)		
IL-6	(+)			(+)		(-)
IL-12p40	(+)		(-)	(+)		

A summary for alterations in cytokine expression in the brain following TMEV infection and restraint stress. The results are shown as the main effect of either infection (INF) or restraint stress (RS), and the interactions between the two variables, which are revealed by ANOVA and post hoc mean comparison. (+) / (-) represent significant increase or decrease (all *p*s < 0.05) in cytokine expression in the brain, respectively.

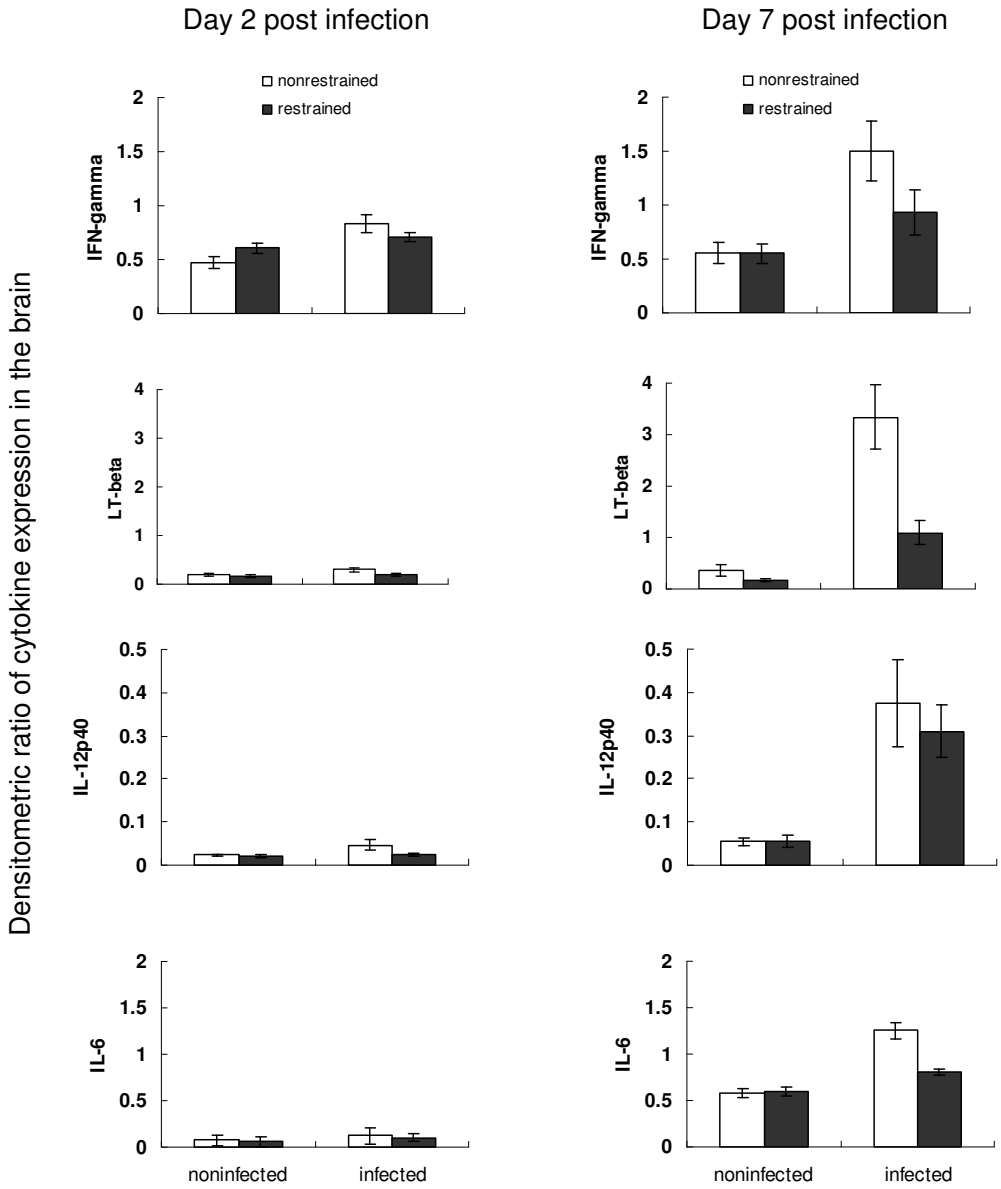

Figure 3-2. The effect of restraint stress on the cytokine expression in the brain during acute TMEV infection. The levels of cytokine expression were derived from the densitometric ratio of the individual cytokine to the housekeeping gene GAPDH and multiplied by 1000. At day 2 p.i., IFN-γ, LT-β, IL-12p40, IL-6 and IFN-β were increased by TMEV infection, and the expression of IFN-γ, LT-β, IL-12p40 were decreased following restraint stress. The cytokines remained at an elevated level at day 7 p.i., and restraint stress reduced the expressions of IFN-γ, LT-β, IL-12p40 and IL-6, however, IFN-β was increased by stress. The values were represented by mean $\pm$ S.E.M.

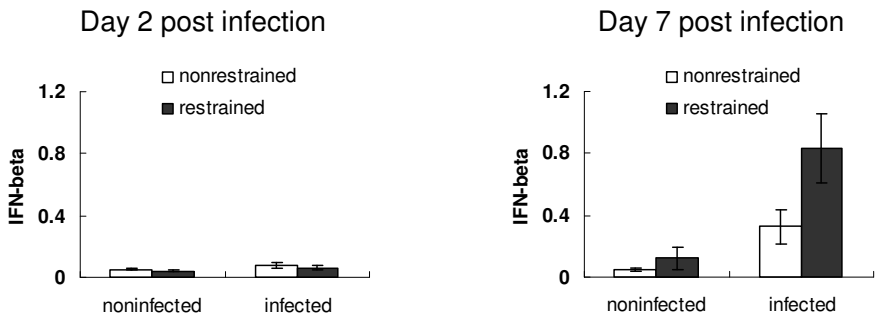

Figure 3-2. continued.

3.2 Cytokine expression in the spleen following Theiler's virus infection and restraint stress

To further examine the effect of restraint stress on the peripheral immune response following infection, we evaluated the cytokine levels in the spleen using RPA at day 2 and 7 p.i. (Figure 3-3).

As summarized in Table 3-2, ANOVA analyses revealed a main effect of infection on cytokine production, wherein Theiler's virus induced increases in TNF-α, TNF-β, IFN-γ, IFN-β and IL-12p40 at day 2 p.i., all *F*s (1,28) > 4.214, *p*s < 0.05 and LT-β, IL-6, IL-1β and IL-12p40 at day 7 p.i., all *F*s (1,58) > 5.033, *p*s < 0.03 (Table 3-2).

A notable effect of stress on the cytokine expression in the spleen was noticed at day 2 p.i., demonstrating that restraint resulted in reductions in TNF-α, TNF-β, LT-β, IFN-γ and IFN-β, all *F*s (1,28) > 7.252, *p*s < 0.01. In contrast, restraint produced a less remarkable suppressive effect of stress on cytokine expression at day 7 p.i. At this time point, only a down-regulation of IFN-β by stress was detected. In addition, stress also elevated a Th2 cytokine, IL-10, in the spleen at day 2 p.i., *F* (1, 28) 5.091, *p*s < 0.03, but this increase did not reach significance at day 7 p.i., *F* (1, 28) = 2.688, *p* = 0.1 (Figure 3-4).

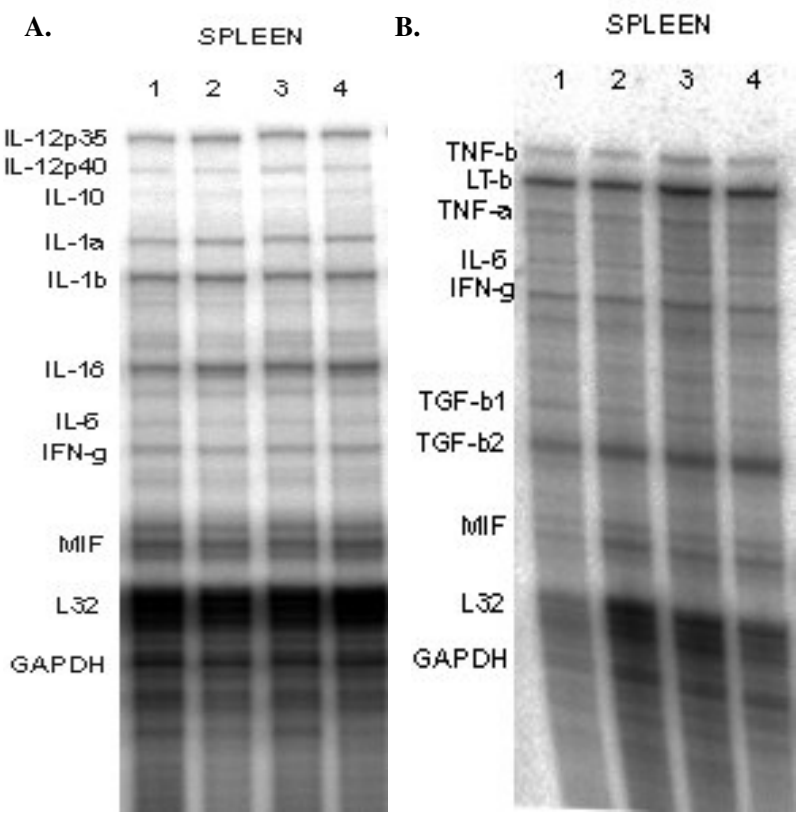

Figure 3-3. Phosphor image of a representative RPA for cytokine expression in the spleen following restraint and/or BeAn infection at day 7 post infection using (A) mCK-2b and (B) mCK-3b kits. Total RNA was isolated from the brains and spleens of CBA mice at day 7 p.i. 25µg RNA was hybridized with a ^{32}P-labeled chemokine probe set as described in Materials and methods. The housekeeping gene L32 was used for the normalization of sample loading and technique error. Lanes 1,2,3,4 represent the brain samples of NI/NR (Non-infected / Non-restrained), NI/R (Non-infected / Restrained), I/NR (Infected / Non-restrained) and I/R (Infected / Restrained), respectively, collected at day 7 p.i..

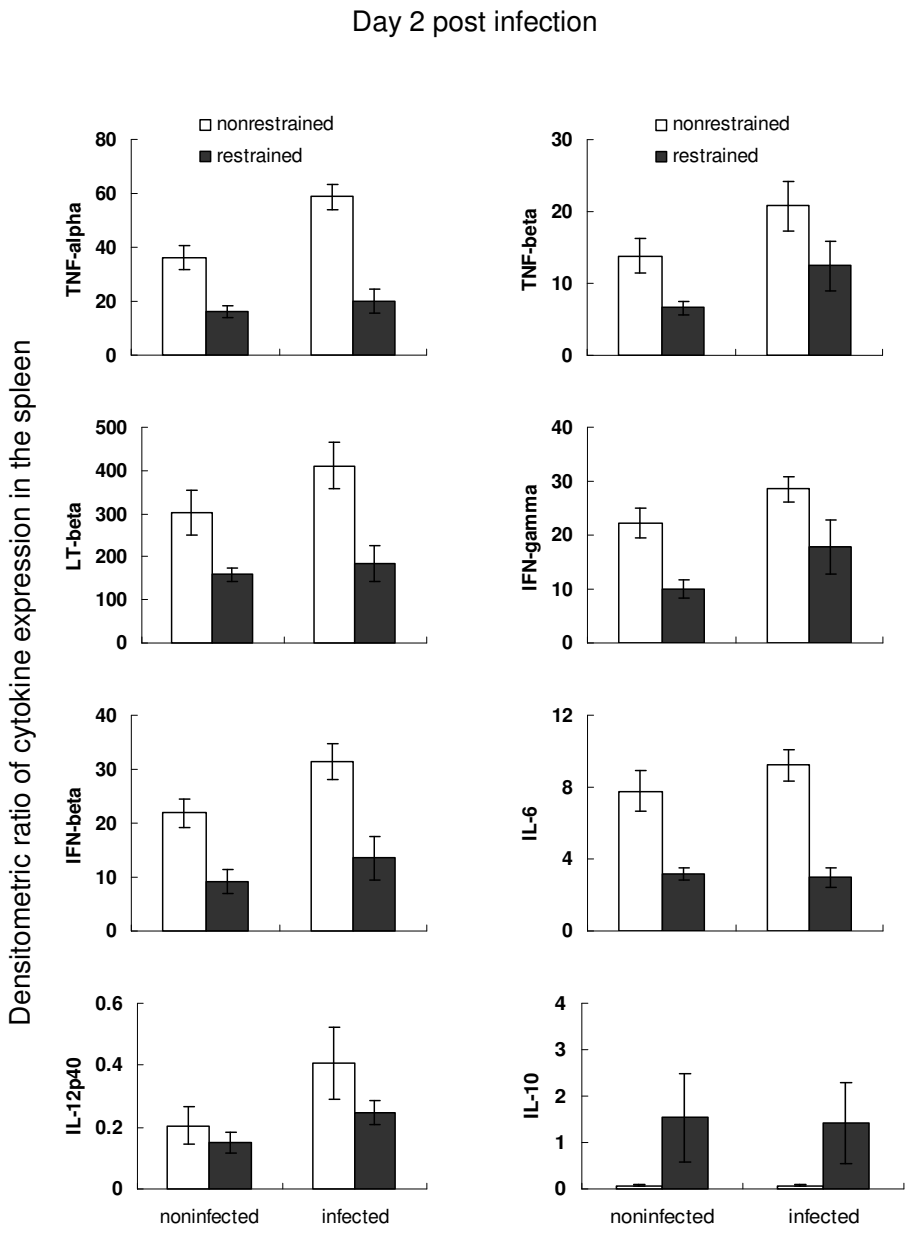

Figure 3-4. The effect of restraint stress on the cytokine expression in the spleen during acute TMEV infection. The levels of cytokine expression were derived from the densitometric ratio of the individual cytokine to the housekeeping gene L32 and multiplied by 1000. At day 2 p.i., restraint stress significantly decreased TMEV-induced expression of proinflammatory cytokines TNF-α, TNF-β, LT-β, IFN-γ, IFN-β, IL-6 and IL-12p40; however, an anti-inflammatory cytokine, IL-10 declined following restraint stress. The values were represented by mean ± S.E.M.

Table 3-2 Summary for alterations in cytokine expression in the spleen following TMEV infection and restraint stress

SPLEEN	Day 2 post infection			Day 7 post infection		
Cytokines	**INF**	**RS**	**INF and RS**	**INF**	**RS**	**INF and RS**
TNF-α	(+)	(-)	(-)			
TNF-β	(+)	(-)	(-)			
LT-β		(-)	(-)	(+)		
IFN-γ	(+)	(-)	(-)			
IFN-β	(+)	(-)	(-)		(-)	
IL-1β				(+)		
IL-6			(-)	(+)	(-)	
IL-10		(+)				
IL-12p40	(+)		(-)	(+)		

A summary of alterations in cytokine expression in the spleen during acute TMEV infection and restraint stress. The results are shown as the main effect of either infection (INF) or restraint stress (RS), and the interactions between the two variables, which are revealed by ANOVA and post hoc mean comparison. (+) / (-) represent significant increase or decrease, all $p<0.05$, in cytokine expression in the brain, respectively.

Restraint decreased the virus-induced cytokine expression of TNF-α at day 2 p.i. and not at day 7 p.i., post hoc mean comparison confirmed significant declines in TNF-β, LT-β, IL-6, IFN-γ and IFN-β in the spleen of I/R mice as compared to I/NR mice at day 2 p.i., all *p*s < 0.05 (Figure 3-3). However, this effect was not observed at day 7 p.i. These results suggest that stress exerts a greater effect on the peripheral immune function by down-regulating pro-inflammatory cytokines during the earlier stage rather than the later stage in restraint animals (Table 3-2).

3.4 The expression of cytokines determined by real time RT-PCR

To confirm the RPA results of cytokine expression, a subset of RNA samples from the brain and spleen at day 7 p.i. was further examined using real time RT-PCR. As summarized in Table 3-3, we confirmed a main effect of infection for TNF-α, LT-β, IL-1β, IFN-γ and IL-12p40 in the brain, and TNF-α, IL-12p40 in the spleen; and a main effect of stress for IFN-γ in the spleen, all *F*s (1,11) > 18.258, *p*s < 0.005. PCR revealed the main effect of infection for IFN-γ in the brain that was not seen in the subset of RPA data but was found in the overall RPA data. In general, the PCR data agreed with the subset RPA data, except for IL-1β and IL-10 in the brain and TNF-α and IFN-γ in the spleen. This discrepancy was also seen when we compared the subset PCR with the overall RPA data, and possibly due to a smaller size (n=11) of samples assayed using PCR than RPA (n=78), resulting a greater variability. Overall, it appeared that a greater agreement of the results was found in the spleen than the brain. Therefore, both methods are sensitive for cytokine detection and the results using either method generally demonstrate a good accordance with each other.

Table 3-3. Comparisons of PCR and RPA on the examination of cytokine expression

Brain (day 7 p.i.)		PCR	
Cytokines	**INF**	**RS**	**INF and RS**
TNF-α	(+)		
Lt-β	(+)	Δ	NI:R>NR; I:R<NR
IFN-γ	(+)		
IL-1β	(+)	(+)Δ	(+)Δ
IL-12p40	(+)		
IL-10	(+)Δ		NI:R>NR; I:R<NR

Brain (day 7 p.i.)		RPA	
Cytokines	**INF**	**RS**	**INF and RS**
TNF-α	(+)		
Lt-β	(+)		*
IFN-γ			
IL-1β	(+)		
IL-12p40	(+)		*
IL-10		(-)	

Spleen (day 7 p.i.)		PCR	
Cytokines	**INF**	**RS**	**INF and RS**
TNF-α	(+)Δ		NI:R<NR; I:R>NR
Lt-β	Δ	(-)Δ	
IFN-γ	(+)Δ	(-)	NI:R<NR; I:R>NR
IL-1β	Δ	(-)Δ	
IL-12p40	(+)		
IL-10		*	

Spleen (day 7 p.i.)		RPA	
Cytokines	**INF**	**RS**	**INF and RS**
TNF-α			*
Lt-β	*	*	*
IFN-γ		*	*
IL-1β	*	*	*
IL-12p40	(+)		
IL-10		*	

(+)/(-) represents an increase or decrease in the levels of expression
* indicates non-statistically significant trends, or patterns of agreement.
Highlighted area indicates the difference between PCR and RPA in a subset of samples
Δ indicates the difference in the results between PCR and overall RPA

3.3 The expression of TNF-α protein level in the brain and serum

TNF-α is an essential cytokine involved in the innate antiviral response, and our previous study showed the serum TNF-α was elevated in all restrained animals at day 1 and 2 p.i., irrespective of whether they were infected or not (Welsh et al., 2004). In the current study, we further examined the CNS TNF-α using western blotting and serum TNF-α using ELISA.

A main effect of infection was observed on the TNF-α level in the brain, F (1,12) = 5.958, $p < 0.04$. An interaction between infection and restraint did not reach significance; however, restraint stress significantly decreased the TNF-α in the brain of infected mice, as confirmed by post hoc mean comparison (Figure 3-5A). The serum TNF-α level was determined by ELISA showing that TMEV infection induced an increase of TNF-α at day 7 p.i., F (1,21) = 9.018, $p = 0.0068$; however, RST did not change the TNF-α level in the serum (Figure 3-5B). The result indicated a difference in TNF-α response in the circulation (systemic) and the CNS (local) following Theiler's virus infection and restraint stress.

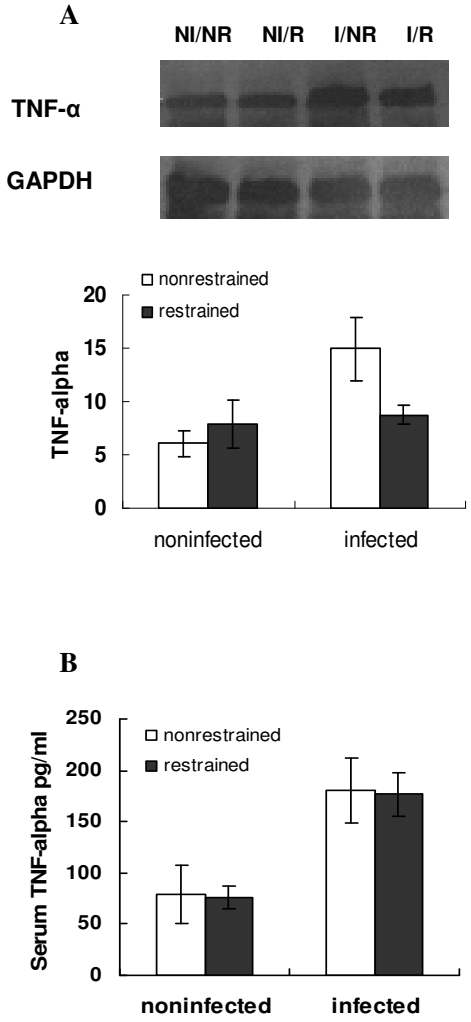

Figure 3-5. The TNF-α protein level in (A) the brain using western blot and in (B) the serum using ELISA at day 7 p.i. (A) The TNF-α protein expression in the brain was increased by TMEV infection, and decreased by restraint stress. The TNF-α level was derived from the densitometric ratio of TNF-α and GAPDH. The value was represented by mean $\pm$ S.E.M. (B) TMEV infection increased serum TNF-α level at day 7 p.i.; however, the level was not affected by restraint stress.

3.4 The correlation between pro-inflammatory cytokine production and viral clearance in the brain

Pro-inflammatory cytokines play an important role in viral clearance and further activation of immune cells in innate immunity to viral infection. To determine whether the level of cytokines correlates with the ability to clear virus, we set up a separate experiment, which consisted of two groups: infected/non-restrained and infected/restrained mice. This allowed us to examine the cytokine expression and the viral titers in the brain from the same animal. At day 7 p.i., the mice were terminated and the brain was dissected out. Then we alternatively took the left or right hemispheres for cytokine assays by RPA and the remaining half for determination of viral titers by plaque assays.

Restraint appeared to impair the viral clearance in the brain at day 7 p.i., resulting in higher viral titers in the restraint mice ($p = 0.001$) as depicted in Figure 3-6. Individual cytokine levels were correlated with viral titers by simple regression. The pro-inflammatory cytokines LT-α, TNF-α and IFN-γ were negatively correlated with viral titers in the brain, wherein higher viral level consistently correlated with lower cytokine expression, all ps < 0.05. In addition, a marginal correlation between the TNF-α protein levels and the viral titers in the brain, wherein an increase in TNF-α was associated with a lower viral titers in the brain, $p = 0.056$, $R = 0.694$ (Figure 3-7). These results indicate the potential role of pro-inflammatory cytokines in viral clearance.

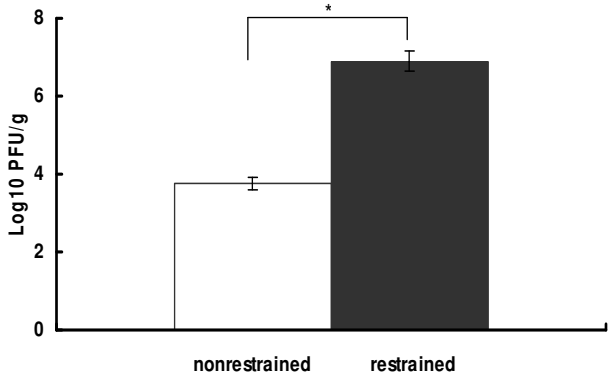

Figure 3-6. The impact of restraint stress on viral titers in the brain at day 7 p.i. by plaque assay. The viral titer was calculated by the logarithm of plaque forming assay (PFU) per gram of brain tissue. The values were represented by mean $\pm$ S.E.M. (*) indicates $p < 0.0001$.

4. Discussion

The current study is a continuation of our investigation of the effects of restraint, as a model for stress, on both the central and peripheral immune response to Theiler's virus infection. Experimental infection of Theiler's virus in the brain elicits both innate immunity and adaptive immunity, which are characterized by CNS mononuclear infiltration consisting of NK cells, macrophages, virus-specific $CD4^+$ and $CD8^+$ T cells, as well as activation of the CNS residential microglia and astrocytes.

4.1 The reduction in cytokine expression suggests suppressed function of immune cells and decreased cellularity following restraint stress

A dominant panel of pro-inflammatory cytokines TNF-α, LT-β, IFN-γ, IFN-β, IL-6 and IL-12p40 was induced in the brain as early as day 2 p.i. and continued to be detected

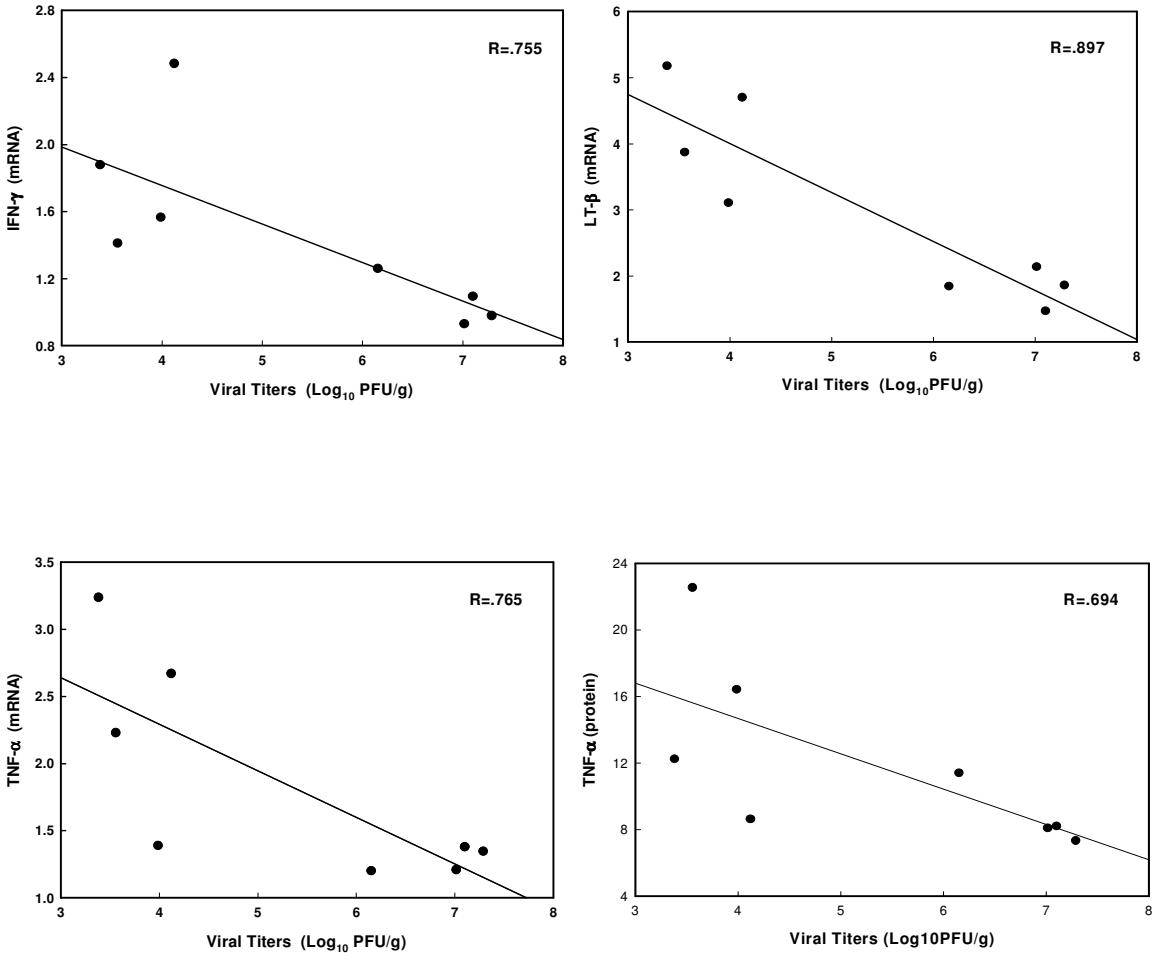

Figure 3-7. The correlation between viral titers and cytokine expression in the brain at day 7 p.i. The viral titers from the left or right hemisphere and the mRNA expression of IFN-γ, LT-β and both the messenger and protein of TNF-α were analyzed by simple regression, R value was indicated in each box, and all the *p*s < 0.05.

up to day 7 p.i., indicative of cellular immunity produced by those potential cellular resources mentioned above. For the first time, we reported a similar profile of pro-inflammatory cytokines TNF-α, TNF-β, IFN-γ, IFN-β, IL-12p40 and IL-6 in the spleen following inoculation of Theiler's virus in the brain, although the profile differed at day 2 p.i. and day 7 p.i. A reduction in the expression of LT-β, IFN-γ, IL-12p40 and IL-6 by restraint was documented in the brain. As compared to the brain, a greater number of cytokines in the spleen, TNF-α, TNF-β, IFN-γ, IFN-β, IL-12p40, IL-6 and LT-β, were profoundly reduced by restraint at day 2 p.i. However, no such effects were observed at day 7 p.i. These results suggest a remarkably suppressive effect of restraint stress on systemic cellular immunity in this model.

IFN-γ, TNF-α and IL-12 are the crucial pro-inflammatory cytokines in the activation of both the innate and adaptive immune components (reviewd in Elenkov and Chrousos, 2002). For example, NK cell activation is critical in the early defense against Theiler's virus infection (Paya et al., 1989). NK cells are regulated by IL-12 and they secrete IFN-γ as one of their immunological effector molecules. Activation of macrophages/microglia to become competent APC is dependent on viral infection, as well as the further stimulation by pro-inflammatory cytokines, such as IFN-γ. IFN-γ, a type II interferon, which has been shown to induce MHC class II expression and various costimulatory molecules, such as B7-1, B7-2, CD40 and ICAM-1 (Olson et al., 2001; Peng et al., 1998), which are required for a functional APC. IL-12 and TNF-α produced by activated APCs promote the cytotoxicity of both NK cells and cytotoxic T cells (CTLs) (Elenkov and Chrousos, 2002; Shaw-Jackson and Michiels, 1997; Watford et al., 2004). The reduced production of these cytokines may reflect a decline in the innate immune functions by

either NK cells, APCs or CTLs following restraint. The down-regulation of NK cell activity and CTL in restraint stress models using a variety of different viruses has been documented by ours and other laboratories (Bonneau et al., 1991; Dobbs et al., 1993; Tseng et al., 2005; Welsh et al., 2004).

The T cell function of the adaptive immune component is regulated by IL-12, a monocyte-derived immunoregulatory cytokine that promotes cellular immunity, particularly in the polarization toward Th1 cytokine production. As a heterodimeric cytokine for IL-12, one of its subunits, IL-12p35, which is constitutively expressed in a variety of cell types, was detected at comparable levels in either the brain or spleen under all the conditions (NI/NR, NI/R, I/NR, I/R); whereas the other biologically active subunit, IL-12p40 was up-regulated at day 2 and 7 p.i., indicating a Th1 cytokine response induced by viral infection. IL-12 and IL-12-induced IFN-γ produced by monocytes may contribute to the Th1 cell differentiation and production of IFN-γ and TNF-β, as well as antagonizing Th2 cell differentiation and the secretion of Th2 cytokines, such as IL-10 (Elenkov and Chrousos, 2002; Watford et al., 2004). We observed a decreased expression of IL-12p40, IFN-γ and TNF-β, as well as an enhancement of IL-10 in the restrained mice at day 2 p.i., indicative of a shift from Th1 toward the Th2 profile and decreased cellular immunity as result of stress.

An increase of IFN-β, a type I IFN, was observed in the brain and spleen following TMEV infection. IFN-β is a potential antiviral cytokine and an inducer of NK cell-mediated cytolysis (Biron, 1999). However, it has also been used as a therapeutic agent for MS and an animal model of MS, EAE, by suppressing both IL-12 production and downstream Th1 cytokines (Klimstra et al., 2000). Unlike other pro-inflammatory

cytokines, IFN-β level was elevated in the brain of I/R mice. Restraint-induced increase in type I IFNs has also been documented in other viral infections, such as influenza virus (Hunzeker et al., 2004). The rise of IFN-β was correlated with impaired viral clearance brought upon by stress at day 7 p.i., which can possibly be explained by a compensatory response to decreased levels of antiviral IFN-γ and other pro-inflammatory cytokines.

In addition to the diminished immune cell activity, which may account for the reduction of cytokine expression, the drop in cellularity of immune cells may also contribute to the change in cytokine levels. It has been shown that stress may alter the cellular composition in circulation and redistribution of immune cells in lymphoid organs using various experimental stressors, such as restraint, immobilization and social stress (Dhabhar et al., 1995; Fleshner et al., 1995; Stefanski et al., 2003). This effect was also observed in the Theiler's virus restraint model by our laboratory, demonstrating that restraint leads to a decreased number of circulating lymphocytes (Welsh et al., 2004). Additionally, we previously showed that stress results in decreased expression of the chemokines, Ltn, IP-10 and RANTES, which are potential chemoattractants for NK cell and T cells (Mi et al., 2004). Therefore, the reduction in cellularity, as well as the decline in recruitment to the inflamed sites following restraint stress, may be additional reasons for reductions in cytokine expression in the brain or spleen.

4.2 The decreased cytokine production is responsible for the impaired viral clearance following restraint stress

Decreased immune function by stress results in impaired viral clearance, as shown by alterations in cytokine expression and the corresponding increase of viral titers in the

CNS of restrained mice. Our results demonstrate a significantly negative correlation between the pro-inflammatory cytokines IFN-γ, lymphotoxin-β (LT-β) and TNF-α and viral load, suggesting a critical role of these cytokines in the containment of TMEV.

IFN-γ is an important immune mediator for viral clearance in the CNS which functions by inducing type II nitric oxide synthase (iNOS) from glia and type I nitric oxide synthase (nNOS) from neurons. IFN-γ has been shown to be critically important in the pathogenesis of several different viral infections, such as measles and herpes simplex virus (Reviewed in Chesler and Reiss, 2002). The crucial role of IFN-γ in Theiler's virus infection has been demonstrated previously, whereby the administration of monoclonal antibody to IFN-γ increases viral persistence and demyelination in both susceptible SJL/J and resistant C57BL/10NJ strains of mice (Rodriguez et al., 1995). Persistent viral infection and demyelination has been also recorded in C57BL/6 IFN-$\gamma^{-/-}$ knockout mice, but not in wild type mice (Murray et al., 2002). In addition, IFN-γ was found to inhibit the in vitro replication of TMEV in cerebral vascular endothelial cell (Welsh et al., 1995).

TNF-α confers antiviral activity, especially when synergized with other cytokines, such as IFNs (Ito and O'Malley, 1987; Wong and Goeddel, 1986). The effect of TNF-α in viral infection of the CNS may be mediated by the upregulation of TNF-α receptor, as reported in an *in vitro* study using mouse hepatitis virus (MHV)-JHM strain (Rempel et al., 2005). The antiviral function of TNF-α has also been demonstrated in cerebral vascular endothelial cell in in vitro TMEV infection (Welsh et al., 1995). In the Theiler's virus model, TNF-α has been shown to play a major role as a toxic effector to oligodendrocytes and consequently demyelination (Selmaj and Raine, 1988). In the acute infection, TNF-α, as well as IFN-γ, is produced by CTL, which mediate the non-cytotoxic

mechanisms, suggesting a potential role for TNF-α in the elimination of virus (Lyman et al., 2004).

LT-β, a membrane form of lymphotoxin expressed on activated T, B and NK cells, plays a critical role in the resistance to intracellular pathogens, including viruses (Benedict et al., 2001; Lin et al., 2003; Marshall et al., 1999; Soderberg et al., 2004), bacteria (Ehlers et al., 2003) and prions (Mabbott et al., 2003; Oldstone, et al., 2002). LT-β induces antiviral activity to Theiler's virus infection by mounting an effective CTL activity enabling viral clearance during the early stage, thus diminishing demyelination at the later stage. Compared to the soluble LT-α, LT-β plays a minor role in demyelination (Lin et al., 2003).

Cytokines alone are not sufficient for complete clearance of virus. However, they are among the core factors that regulate or coordinate the immune cells, and changes in their level may be indicative of the degree of activation of immune system.

4.3 Alteration in cytokines expression is mediated by both humoral and neuronal mechnisms

It is well recognized that glucocorticoid regulates the Th_1/Th_2 balance by suppressing IL-12, thus removing its inhibitory effect on the production of Th_2 cytokines, such as IL-10 (Elenkov and Chrousos, 2002). This is supported by the finding that in the restrained animals, IL-12p40 expression was significant lower, and IL-10 was elevated as compared to that in the non-restrained animals. The turnover of IL-12/IL-10 could be explained by restraint-induced elevation of circulating corticostrone as reported previously (Campbell et al., 2001). *In vitro* studies revealed that the suppressive effect on IL-12 by

dexamethasone is mediated by inhibition of mitogen-activated protein kinase (MAPK) and transcription factor NF-κB (Ma et al., 2004) or by decreased expression of IL-12 receptors β_1, β_2 and IL-12 binding ability (Wu et al., 1998). It has also been reported that glucocorticoid induces IL-10 production, as well as other Th_2 cytokines, IL-4 and IL-13 in rat $CD4^+$ T cells (Ramierz et al., 1996). Recently, another type of IL-10 producing T cell, known as T regulatory cell (T_R 1 cell), was shown as a potential source of IL-10 following glucocorticoid exposure, which is mediated by upregulation of a different transcription factor, FOXP3 (Karagiannidis et al., 2004). Moreover, glucocorticoid can promote IL-10 production by both macrophages and dendritic cells (Franchimont, 2004); thus, together with the T_R 1 cell, these cells may be the major source of IL-10 in the restraint response.

An IL-10 increase, which is a Th_2 response, was only observed in the spleen, not in the brain in our model. We speculate that restraint-induced catacholamines (CAs) may be involved in this Th1/Th2 regulation, in addition to the hypothesis of peripheral-derived immune regulation. Because the spleen is predominantly innervated by sympathetic nerve fibers, activation of the SNS results in the release of norepinepherine in either a synaptic or a nonsynaptic manner in the spleen (Elenkov et al., 2000), for example, during restraint stress. Catecholamines inhibit IL-12 and subsequent Th1 cytokine productions but stimulate IL-10 production via β_2-adrenergic receptors, thus providing additional machinery for the Th_1/Th_2 transition (Elenkov and Chrousos, 2002). The Th2 skew was observed at day 2 p.i. but not day 7 p.i., which was in parallel with the finding that a greater number of Th_1 cytokines were reduced by restraint stress. It is possible that the repeated restraint of eight sessions may produce an adaptation to the stressor after the

first two or three sessions of restraint, as evidenced by behavioral observations (data not shown), so that a blunted SNS response or glucocorticoid resistance in the spleen might occur due to the extended stress exposure. However, the corticosterone which was sustained at elevated levels over the entire session of restraint (unpublished data) may contribute to the major suppressive effect of stress. By contrast, another study showed that neither splenic nerve transection nor adrenalectomies (ADX) can abrogate the increase in foot shock-induced cytokine production in the spleen (Meltzer et al., 2004). Therefore, it is conceivable that both the neural and hormonal mechanisms are necessary for induction of the stress effect.

4.4 Differential immunoregulatory mechanisms may be involved in the CNS and peripheral immune organs during stress

Generally, our data indicate similar but distinct cytokine profiles in the brain and the spleen following infection, with a greater number of Th_1 cytokines detected in the brain. This could be due to several mechanisms, including either more potential cellular sources for cytokine production in the brain, such as astrocytes, microglia and infiltrating inflammatory cells; or a higher viral load in the CNS than in the spleen (manuscript submitted).

The discrepancy between immune response in the brain and spleen is further demonstrated by the differential expression of IL-10 in the two organs. The increase in IL-10 caused by stress was only found in the spleen at day 2 p.i., and not at day 7 p.i.. The elevation of IL-10 appears to be associated with the decline in Th1 cytokines and IL-12 production in the spleen. The IL-10 response was never detected in the brain. However,

the down-regulation of IL-12p40 and Th_1 cytokines were present up to day 7 p.i. in the brain. It is plausible that two mechanisms may account for the reduced Th_1 response following stress: either by reducing the expression of the upstream cytokine, IL-12p40; or by producing the Th_1-inhibitory cytokine, IL-10. However, the skew in Th_2 response was only noticed in the spleen at day 2 p.i., suggesting the inhibitive role of Th_2 cytokine on Th_1 cytokine production was transient in this restraint model. The absence of Th_2 response in the brain at either time point examined, may suggest an additional mechanism, that a direct down-regulation of Th1 cytokines, rather than inhibition by Th_2 cytokines, plays a major role in the stress-induced immune response to Theiler's virus infection. We speculate that a regulatory effect by Th_2 cytokines during restraint stress may occur in the periphery.

IL-1β is a pleiotropic pro-inflammatory cytokine that is produced in response to various stimuli, such as infection and stress. IL-1β was expressed following infection, but not induced by stress, in the brain in this restraint model. Indeed, Deak et al. (2004) reported that the hypothalamic IL-1β production, which is the major CNS source for IL-1β during stress response, is induced by hypoglycemia, but not by restraint. Therefore, this may indicate that the IL-1 production in the CNS is dependent on the type of stressor used at exposure. Several other cytokines were found differentially expressed in the brain and spleen following stress and/or infection, such as TNF-β and IFN-β, a further indication of the difference in the immune response between the CNS and peripheral lymphoid organ.

In summary, restraint stress of mice infected with Theiler's virus, produced decreased pro-inflammatory cytokine expression in the brain and spleen, as compared to

the non-restrained mice, and caused impaired viral clearance and subsequent prolonged or persistent viral infection. The stress-induced regulatory mechanisms in viral infection and the cell type involved in the process warrant further investigation.

IV THE EFFECT OF RESTRAINT STRESS ON THE SYSTEMIC TMEV INFECTION

1. Introduction

Psychological or physiological stress has been associated with compromised immune function and is implicated in the onset, reactivation or exacerbation of a number of infectious and autoimmune diseases. The response to a stressor, defined as a challenge that could alter or disturb the stability of the internal milieu, is characterized by the release of stress hormones (e.g. glucocorticoids) and neurotransmitters (e.g. catecholamines) due to the activation of the hypothalamic-pituitary-adrenal (HPA) axis and the systemic/adrenomedullary sympathetic nervous system (SNS) (Chrousos & Elenkov, 2002; Stratakis & Chrousos, 1995). Glucocorticoids have long been known to be anti-inflammatory and immunosuppressive (Barnes & Adcock, 1993), and play a vital role in the stress-induced immune response.

Restraint stress (RST), induced by restricting the animal's movement, has served as a common procedure for studying stress effects in laboratory animals (Pare & Glavin, 1986). The stress-induced immunosuppression in viral infections has been extensively investigated. Studies have demonstrated that stress suppresses infiltration of mononuclear cells (Sheridan et al., 1991); production of virus specific cytokines (Bonneau, 1996; Sheridan et al., 1991); and CTL responses (Bonneau et al., 1991; Bonneau et al., 1993; Wonnacott & Bonneau, 2002). Consequently, viruses may establish persistent infections as a result of the impaired immunological surveillance.

Theiler's murine encephalomyelitis virus (TMEV) is a member of the cardiovirus

genus of the family Picornaviridae. It is a naturally occurring, murine enteric pathogen causing asymptomatic infection of mice, and occasionally, it penetrates into the central nervous system (CNS) resulting in a CNS disease (Theiler, 1934). Experimental intracranial inoculation of the BeAn or DA strains of TMEV into susceptible mice induces a biphasic disease - an acute encephalomyelitis in the gray matter and a chronic progressive demyelination involving the white matter of the spinal cord (Lipton, 1975). The chronic disease, both clinically and microscopically resembling human multiple sclerosis (MS), has been used as an important experimental model for MS.

Upon initial infection, TMEV predominantly replicates in neurons (Brahic, et al., 1981), and then spreads to macrophages and glial cells in the CNS (Clatch et al., 1990; Dal Canto & Lipton, 1982; Lipton et al., 1995; O'Shea et al., 1997). For mice susceptible to demyelination, the viral RNA can be detected within the CNS as late as one year p.i.. Furthermore, TMEV results in systemic infection as evidenced by viral RNA detection in the heart, intestine and lymph nodes of infected mice, but can be eliminated by four months p.i. (Trottier et al., 2002). In contrast, resistant mice clear the virus from CNS in the acute phase and do not develop demyelination (Dal Canto & Lipton, 1975; Lipton, 1975), which emphasizes the necessity for TMEV to establish a persistent infection during the early phase in order to induce demyelination at the late stage of disease.

The establishment of viral persistence is determined by the genetic characteristics of the mice. For susceptibility to TMEV persistence and demyelination, the major genetic basis was mapped on H-2D^{d} locus, whereas the resistance was linked to H-2D^{b} locus (Brahic & Bureau, 1998); although other genes, such as *Tmevp2, Tmevp3*, have also been implicated (Bihl et al., 1999). In addition, a timely, strong immune response by the host

is critical for clearing the virus from CNS. During the early disease, NK cells, macrophages, and virus-specific $CD4^+$ and $CD8^+$ CTL are recruited into the CNS and contribute to the elimination of TMEV from the CNS (Kang, et al., 2002; Murray, et al., 2002; Oleszak et al., 2004; Paya, et al., 1989). In addition, CNS $CD4^+$ T infiltrates participate in clearing the virus by generating Th1 cytokines, rendering help for the $CD8^+$ response, as well as promoting the humoral immune response (Karls et al., 2002; Sato, et al., 1997, Welsh et al., 1987).

However, the effectiveness of viral clearance can be undermined by an impaired immune response. For example, resistant BALB/cByJ mice became susceptible to TMEV following depletion of T cells by experimental exposure to low-dose irradiation (Nicholson et al., 1996). Also, an administration of immunosuppressive cyclophosphamide and rabbit anti-mouse thymocyte serum to TMEV-susceptible SJL/J mice (Lipton et al., 1977) and anti-CD4 monoclonal antibody to CBA mice (Welsh et al., 1987) resulted in higher mortality, a prolonged high level of virus, and an increase in the severity of CNS injury.

We previously reported that the RST in CBA mice resulted in impaired viral clearance and attenuated inflammation in the CNS, as well as worsening clinical symptoms and higher mortality during the acute phase, in part, by elevating circulating corticosterone (Campbell et al., 2001), decreasing NK cell activity (Welsh et al., 2004), and down-regulating chemokine expression (Mi et al., 2004). In the current series of experiments, we investigated the effect of stress on the viral clearance of TMEV and the pathology in systemic organs. We hypothesized that immunosuppression by RS could exacerbate systemic TMEV infection, thus facilitate the establishment of viral persistence.

Additionally, since TMEV displayed preference for myocardium and skeletal muscle (Gomez et al., 1996), we further examined whether restraint stress resulted in any change in pathogenecity of TMEV to particular organs.

2. Materials and methods

2.1 Experimental design

Three experiments were conducted in this study using separate groups of mice.

1) Experiment 1. Determination of viral titers in the CNS and extraneural organs. All the mice were infected with BeAn. A 2 (restraint vs. non-restraint) by 3 (time of sacrifice days 1, 3 and 7 p.i.) factorial design, 3-10 mice per cell, were used to determine the impact of RS on viral titers in organs throughout the body.

2) Experiment 2. The pathology in systemic organs following infection and RS. A 2 (infected vs. non-infected) by 2 (restraint vs. non-restraint) factorial design, 3-6 mice per cell, was applied to examine the microscopic pathology in systemic organs. All the mice in this experiment were sacrificed at day 7 p.i.

3) Experiment 3. TMEV pathogenecity following stress. To investigate whether RST can alter the pathogenecity of TMEV, we isolated the virus from the heart of infected/restraint mice from experiment 1, and intracranially inoculated the isolates to a third group of mice. A 3 (virus isolates from the hearts of 3 infected/restraint mice) by 3 (time of sacrifice days 1, 3 and 7 p.i.) factorial design, 3-4 mice per cell, but no restraint was applied.

2.2 Mice

Male CBA mice at three weeks of age were obtained from Harlan Labs, Indianapolis, IN. Mice were maintained under a pathogen-free environment in a 12:12 light/dark cycle (lights on from 6:00AM to 6:00PM). Mice were counterbalanced by weight upon arrival, and housed 3-4 mice per cage. Mice were allowed to acclimate to such conditions for one week prior to any experimental manipulations.

At four weeks of age, cages of mice were randomly allocated to one of the following experimental conditions, if applied, dependent on the experimental design: (1) NI/NR: mice remained undisturbed in their home cages; (2) NI/R: each mouse was subjected to RST in their home cages; (3) I/NR: mice were infected intracerebrally with BeAn virus; or (4) I/R: each mouse was intracerebrally infected with BeAn virus and subjected to RST.

2.3 Virus

The BeAn strain of TMEV was kindly provided by Dr. H. L. Lipton, Department of Neurology, University of Chicago, Evanston, IL. The virus was propagated and titrated on L2 cells by standard plaque assay and stored at -80^{o}C (Welsh et al., 1987).

2.4 Restraint stress protocol

Mice were physically restrained in a 60 ml polypropylene syringe, which was perforated with ventilation holes of 0.4 cm diameter. Mice were restraint stressed for 12 h (9pm-9am) daily. Restraint stress was initiated one day prior to infection, and continued for seven more consecutive nights (Campbell et al., 2001; Sheridan et al., 1991).

2.5 Infection of mice

Mice were anesthetized with isoflurane (Rhoda Organique Fine Ltd., Avonmouth, Bristol, UK) and $5.5X10^4$ PFU BeAn virus in 20 µl inoculum was injected intracranially into the right cerebral hemisphere. Animals in non-infected groups were sham-infected with 20 µl sterile phosphate-buffered saline (PBS).

2.6 Determination of viral titers

The brain, spinal cord, thymus, spleen, superficial cervical lymph nodes (SCLN), deep cervical lymph nodes (DCLN), lumbar lymph nodes (LLN), mesenteric lymph nodes (MLN), heart, lungs, liver, intestine, kidneys and testes were aseptically removed, weighed, and homogenized in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen Corporation, USA). Cellular debris was centrifuged and the titer of virus in the supernatant was determined by standard plaque assays on L2 cells.

2.7 Collection of tissue for microscopic examination

In experiment 2, the mice were sacrificed at day 7 p.i. The organs, as described above, were removed, fixed in 10% formalin, embedded, sectioned and stained with hematoxylin & eosin (H&E) for microscopic examination.

2.8 Recovery and amplification of heart-adapted TMEV

A previous study showed that TMEV exhibited a predilection for the heart and skeletal muscle when injected intraperitoneally, causing myocarditis and myositis in susceptible strains of mice and immunodeficient SCID mice (Gomez, 1996). To

investigate if stress alters the pathogenecity of Theiler's virus to myocardiocytes, resulting in increased viral replication and lesions in the heart and the other organs, 3 infected/restrained mice, which were terminated at day 7 p.i., were randomly selected for this study.

Virus from the heart homogenates of the 3 mice was amplified in L2 cells. Briefly, the monolayer of L2 cells in a T-25 flask were incubated with the virus-containing supernatant in the presence of DMEM and 0.5% BSA at room temperature for 1h. Then the medium was removed and the cells were cultured with DMEM-0.5% BSA at 37^0C and 3% CO_2 for 2 –3 days until a cytopathic effect (CPE) was seen microscopically. The monolayers were then removed with a scraper and transferred with the medium to a centrifuge tube. After centrifuging at 2000 rpm, 4^0C, the supernatant was incubated on L2 monolayers in a T-75 flask at the same condition as described above. The virus was further amplified in a T-125 flask following the same procedures as above when CPE was observed. The virus-containing supernatant was collected and the titer of the virus was determined by plaque assay.

Three isolates of virus ($5.5X10^4$ PFU/g) propagated from the hearts were intracranially injected into three groups of non-restrained CBA mice. The mice were sacrificed at days 1, 3, 7 p.i. The brain, heart, thymus, spleen, gastracnemius muscle were aseptically removed. In order to determine whether there was a correlation of viral load with the histopathological changes, half of the tissues were processed for viral titration by plaque assays, and the other half of the tissues were processed for histopathology. Additionally, the sciatic nerves were also dissected for histopathological examination.

2.9. Validation of recovered virus as Theiler's virus by radioimmunoassay (RIA)

In order to determine that the viruses isolated from the heart were indeed Theiler's virus, their identity was confirmed using an RIA as previously described (Sieve et al., 2004). Briefly, a flexible u-shaped, 96-well polyvinyl chloride plate (Costar, Cambridge, MA) was initially washed with PBS/Tween-20 (0.05% v/v) and rinsed by reverse osmosis (RO H_2O). The plate was coated with 100 µL of supernatant from each sample (1.0 x 10^7 pfu/100 µL) and incubated at 4°C for 24 hours. Following the incubation, the plate was washed as described above. The plate was blocked with 3.0% non-fat dry milk (NFDM) in PBS (pH 9.0) for one hour at 37°C. After washing, either a positive control serum from rabbit (generated by 3 serial injections of UV inactivated BeAn strain of Theiler's virus into a rabbit), or an anti-goat negative control serum from rabbit (Sigma, Saint Louis Missouri, USA) was diluted 1/20 in assay buffer (made up from two parts: 495 mL of part A: 0.08M Trizma HCl, 0.03M Trizma base and 0.15M NaCl at a final pH of 7.2, and 5 mL of part B: 1.0% NFDM and 0.5% Tween-20 in RO H_2O) and added to the wells (100 µL total volume) . Serial two fold dilutions of antiserum were diluted to 1/640 of the original concentration. Following the serial dilutions, the plate was then incubated for one hour at 37°C. Next, the plate was washed and rinsed again (as previously described) before adding 100 µl polyclonal goat anti-rabbit (Sigma, Saint Louis, Missouri, USA) antibody diluted 1/500 in assay buffer to every well. After incubating at 37°C for one hour and a subsequent washing, 100 µl of ^{125}I-Protein A (1 x 10^5 cpm/100 µl assay buffer) was added to each well, and the plate was incubated at room temperature for one hour. The plate was then washed and rinsed with PBS/Tween-20 and RO water (as described above). Once the plate was dry, every well was cut out, put into tubes and

counts were determined by using a gamma counter (Beckman gamma 5500 counting system).

3. Results

3.1 Effects of stress on viral titers in organs

3.1.1 The stress effects on the viral titers in the CNS

During the first seven days p.i., stress resulted in a significant increase in the overall viral titers in the brain F (1,23) = 4.407, $p < 0.05$, but not in the spinal cord, F (1,23) = 1.172, $p > 0.05$. The viral titers in both the brain and the spinal cord increased with the day p.i., all Fs (1,23) > 7.331, ps < 0.003. The main effect of stress on viral replication was not seen until day 7 p.i, when the stressed mice were developed significantly higher titers in the brain and the spinal cord than the non-restrained mice, all Fs (1,15) > 8.376, ps < 0.01 (Figure 4-1).

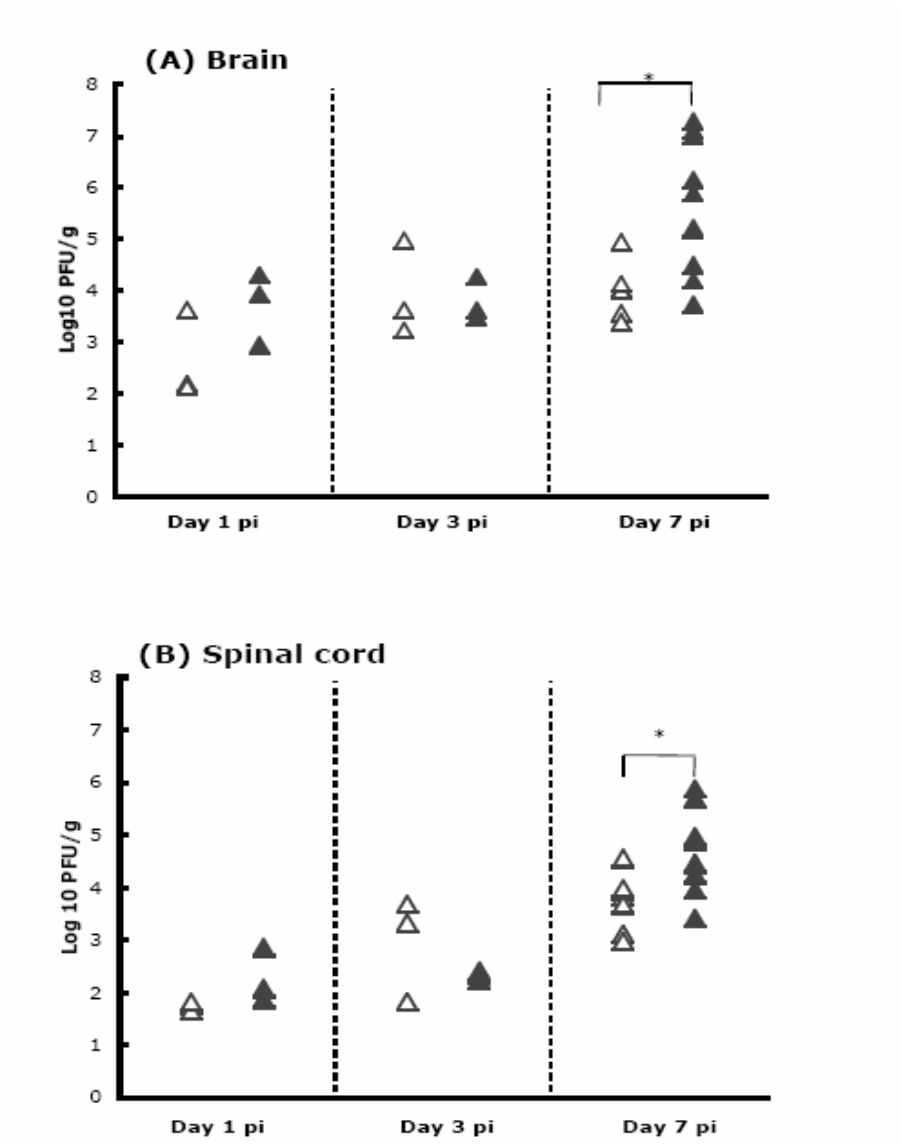

Figure 4-1. The effects of restraint stress on TMEV replication in the brain **(A)** and the spinal cord **(B)** during early infection. The viral titers are represented by logarithmic plaque forming unit per gram of tissue examined. Δ and ▲ indicate non-restraint and restraint mice, respectively. The number of mice used with each experimental condition ranged from 3-10 animals. * indicates a significant difference in viral titers between restrained and non-restrained mice, $p < 0.05$.

3.1.2 The effect of stress on the viral titers in extraneural organs

Although TMEV is mainly used in the study of neurological disease, it was originally identified as an enteric pathogen, and there is also evidence that this virus may systemically infect other organs (Gomez et al., 1996). In this experiment, extraneural organs were also removed from the animals to determine whether stress altered viral dissemination.

Our results showed that stress increased viral titers in the heart [F (1,15) > 13.17, p < 0.01], and a timing effect of enhanced replication of virus over time [F (1,15) > 13.71, p < 0.001]. The infectious virus was only detected in the heart in 1 out of 3 (33.3%) non-restrained mice as late as day 7 p.i. However, in the restrained mice, the replication of TMEV was observed in 2 out of 3 (66.7%) at day 3 p.i. and all 6 mice (100%) at day 7 p.i.; moreover, the titers were increased in the restrained mice at day 7 p.i. [F (1,7) > 12.82, p < 0.01] (Figure 4-2A).

The virus was occasionally found in the lungs, liver and intestine of restrained mice but not in none of the non-restrained mice during the first 7 days p.i., suggesting that restraint tended to facilitate the propagation of virus in these organs, although difference were not statistically significant due to insufficient animals (data not shown). The virus was never detected in the kidneys or testes in either restrained nor non-restrained mice.

Lymphoid organs are particularly important tissues to examine during viral infection. Consequently, three groups of local lymph nodes (LNs), including cervical LNs, mesenteric LNs and lumbar LNs, spleen as well as thymus were examined. It appeared that restraint increased the overall viral titers in cervical LNs and mesenteric LNs during

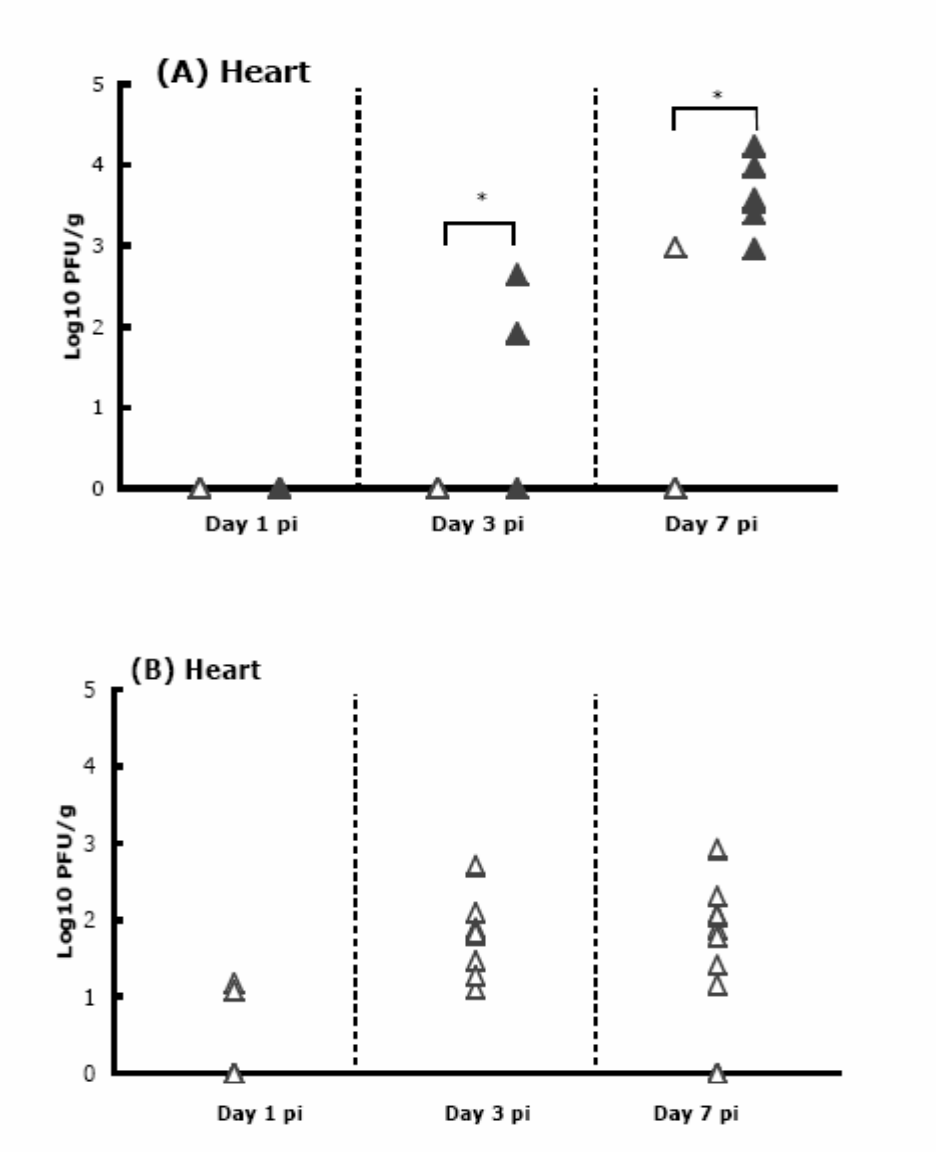

Figure 4-2. **(A)** The effects of restraint stress on parental TMEV replication in the heart during early infection. The data was derived from 3-6 animals per group **(B)** The effects of restraint stress on the pathogenecity of TMEV to the heart. The data was obtained from 9-12 mice per group, which were infected with heart-adapted TMEV. The viral titers are represented by logarithmic plaque forming unit per gram of tissue examined. Δ and ▲ indicate non-restrained and restrained mice respectively. * indicates a significant difference in viral titers between restrained and non-restrained mice, $p < 0.05$.

the 7 days post infection, all *F*s (1,14) > 5.897, all *p*s < 0.05. Compared to the cervical LNs (10^4 PFU/g), lower viral loads (10^3 PFU/g) were exclusively identified in restrained mice, from which the virus was detected in the mesenteric LNs of 3 out of 3 mice at day 3 p.i. and 3 out of 6 mice at day 7 p.i., and in the lumbar LNs of 4 out of 6 mice at day 7 p.i. (Figure 4-3). However, these stress effects were not significant [*F*s (1,7) < 4.264, *p* > 0.07] except in the mesenteric LN at day 3 p.i with F (1,4) = 56.58, p = 0.002.

Stress appears to enhance the overall viral replication in the spleen during the first 7 days p.i., [F(1,15) > 16.96, p < 0.001]. Restraint resulted in an increase in viral load in the spleen as early as days 1 p.i., as well as at day 3 p.i. [*F*s(1,4) >11.604, *p*s < 0.05]; however, a longer stressor of 8 nights did not impact the viral clearance in the spleen, F (1,7) = 0.21, p = 0.65. This finding was further confirmed by ANOVA showing an interaction between stress and the duration of stress on viral replication, which indicated a suppressive effect of stress on viral amplification at days 1 and 3 p.i. [F(1.15) = 7.119, p = 0.0067], but not day 7 p.i. (Figure 4-3).

Although the thymus appeared not to be a preferential site for TMEV replication under non-restrained condition, our results showed a potention that infection could be enhanced by a longer duration of stress of 8 nights, in 2 of 6 restrained mice, with a viral titer of 10^3~10^4 PFU/g. However, this did not reach significance (Figure 4-3).

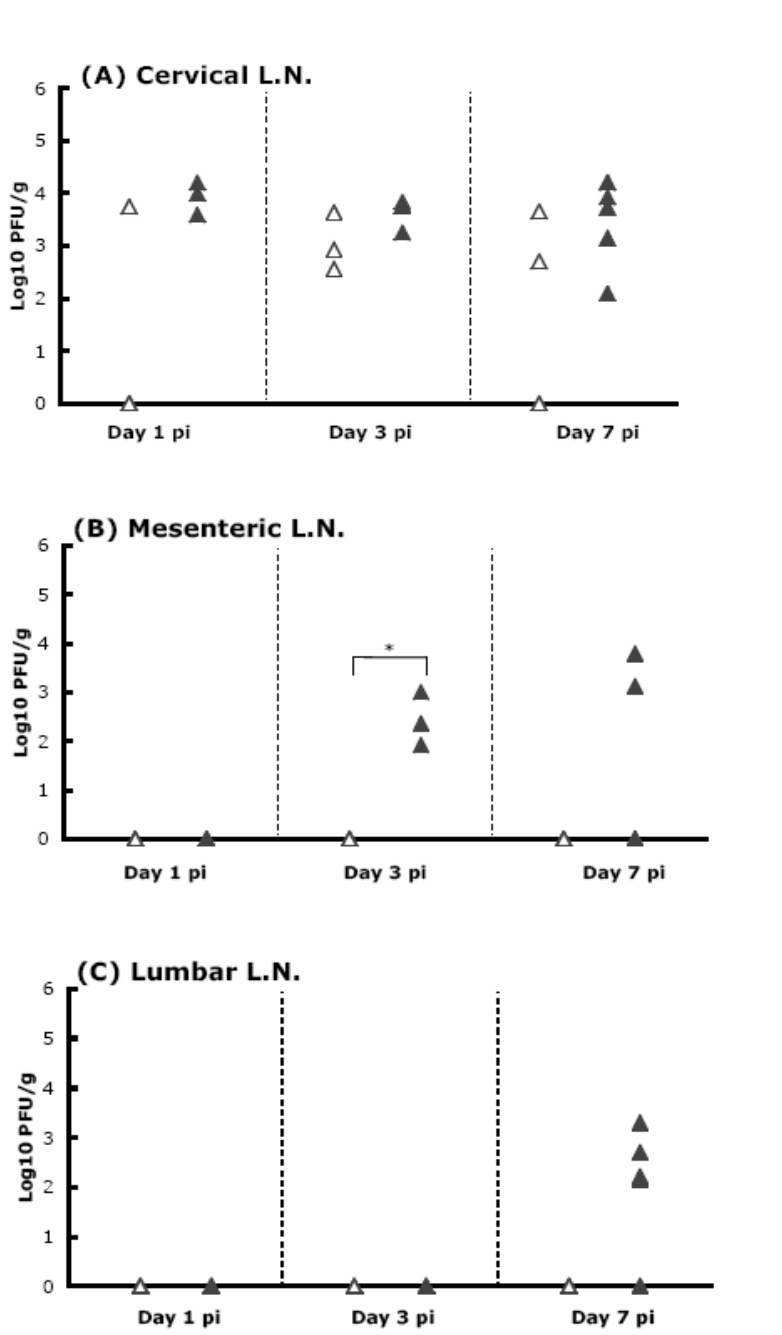

Figure 4-3. The differential effects of restraint stress on TMEV replication in lymph nodes (LN): **(A)** cervical LN, **(B)** mesenteric LN, **(C)** lumbar LN and lymphoid organs: **(D)** spleen and **(E)** thymus during early infection. The viral titers are represented by logarithmic plaque forming unit per gram of tissue examined. Δ and ▲ indicate non-restraint and restraint mice respectively. The number of mice used in each experimental condition ranged from 3-6 animals per group. * indicates a significant difference in viral titers between restrained and non-restrained mice, $p < 0.05$.

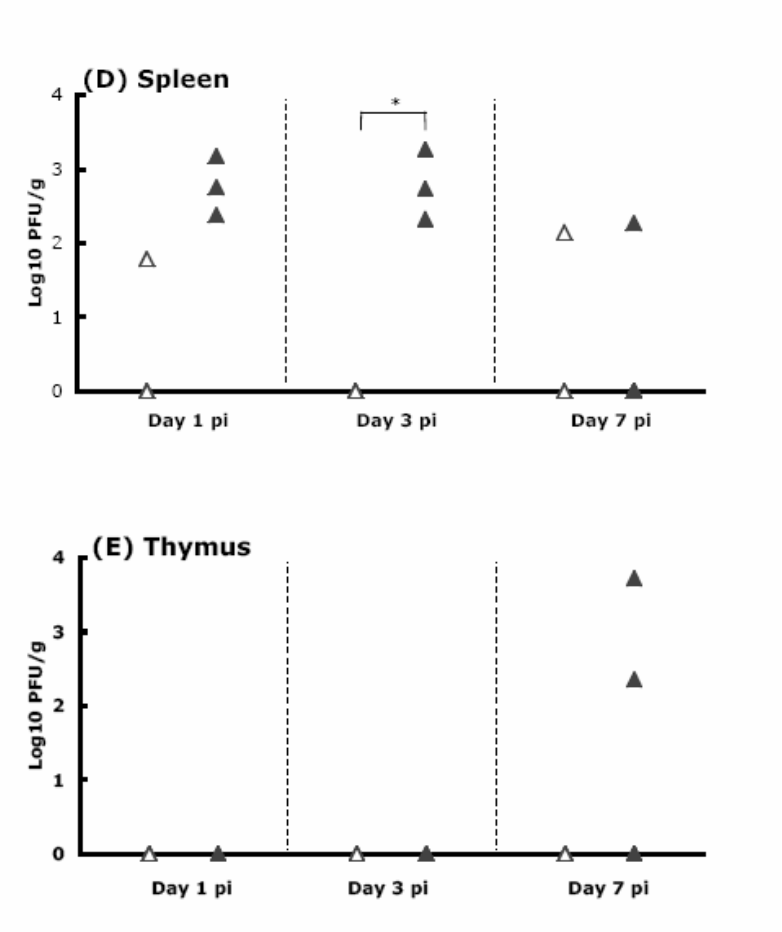

Figure 4-3. continued.

3.2 Histopathology

3.2.1 CNS changes following stress and infection

At day 7 p.i. with TMEV virus, a non-suppurative inflammation characterized by perivascular cuffing, microgliosis and meningitis, was present in I/NR mice; whereas a reduced pattern of inflammation was observed in the brain of I/R animals, that was consistent with our previous findings (Campbell et al., 2001; Mi et al., 2004).

Inflammation, evidenced by cuffings and microgliosis, not as remarkable as seen in the brain, primarily involved the gray matter of the cervical, thoracic and lumbar spinal cord at day 7 p.i. in I/NR mice (Figure 4-4A). A mild meningitis, cuffs and microgliosis also occurred in the preceding areas of the spinal cord (Figure 4-4A, A1,A2,A3,A4). No lesions were detected in the sacral spinal cord. In contrast with the observations in I/NR mice, I/R mice had little evidence of inflammation in the spinal cord (not shown).

3.2.2 The response of lymphoid organs to stress and infection

The thymuses of both I/R and NI/R mice were markedly reduced in volume with depletion of T lymphocytes that caused a pronounced alteration of lobular architecture and a loss of corticomedullary distinction. Although the medulla remained identifiable, it contained few T lymphocytes but an increase in adipose tissue (Figure 4-4B, C). No lesions such as inflammation, indicating viral infection, were detected in infected mice.

Restraint stress also greatly reduced the size of the spleen in either infected or non-infected animals. However, no lesions were detected in the spleen or lymph nodes microscopically.

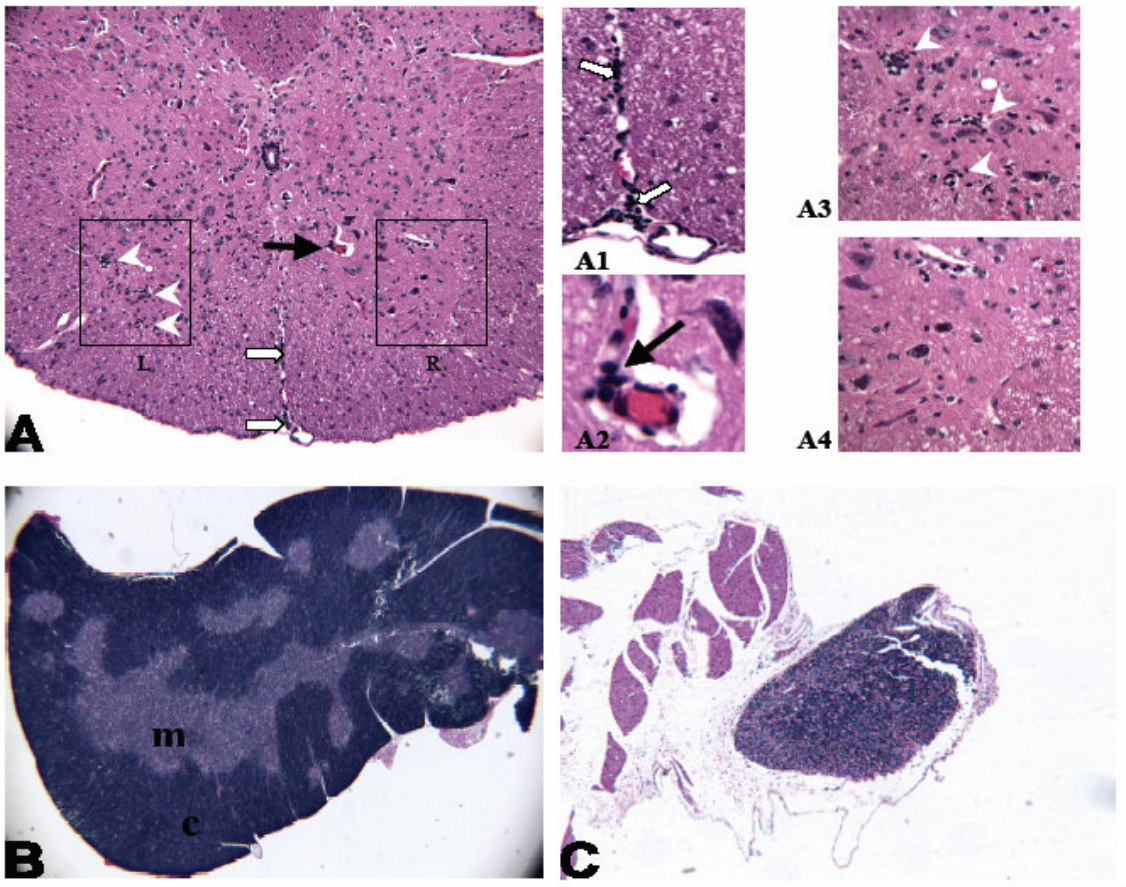

Figure 4-4. The effects of restraint stress on the histopathological changes of systemic organs by H&E. (**A**) A section of spinal cord of an infected/non-restrained mouse demonstrated a mild inflammation (20X) represented by meningitis (shown as white arrows in A and A1 at 40X magnification), peripheral vascular cuffings (indicated by black arrow in A and A2 (40X)), and microgliosis (MG) as indicated by white arrow heads in the box at left (L) of A and A3 (40X). The counterpart area at the right (R) in the spinal cord showed no microgliosis as seen in A4.
(**B**) The thymus from a normal mouse (4X) showed cortex (c) and medulla (m). (**C**) The thymus from a restrained mouse. (4X).

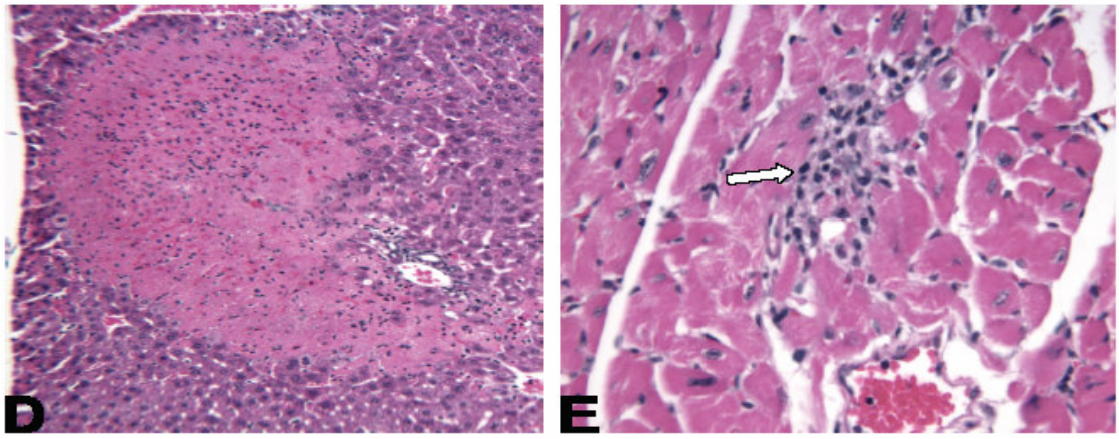

Figure 4-4. continued. (**D**) A representative liver section of restraint mice showed severe hemorrhagic hepatic necrosis (10X). (**E**) Restraint stress on the pathogenecity in the heart of Theiler's virus. A mild inflammation as indicated by white arrow in myocardium was observed in a mouse intracerebrally injected with heart-adapted TMEV.

3.2.3 The pathology in systemic organs after infection and stress

A focal subcapsular hepatic necrosis occurred in restrained (both infected and non-infected) mice, as seen in Figure 4-4D. The lesion was characterized by coagulation necrosis with various degrees of hepatocellular degeneration and fragmentation, scattered accumulation of neutrophils and presence of mild hemorrhage. A mild focal hepatocellular vacuolation adjacent to the necrosis area was also present. A Gram staining indicated no bacterial infection of the liver was identified due to RST.

No lesions were detected in the kidneys, testes, intestines, heart or lungs of either I/R or I/NR mice, despite the identification of virus in the latter two organs of I/R animals.

3.3 The effect of stress on viral pathogenecity

3.3.1 The viral replication of heart-adapted virus in the brain, spleen, thymus, heart and skeletal muscle

We have shown that stress altered the susceptibility of a variety of tissues or organs to TMEV infection. In order to further confirm that the pathogenicity of TMEV could be altered by stress, the virus isolated from heart homogenates, designated as heart-adapted virus (HAV), of three restrained mice was propagated (as described in Materials and methods), collected and injected intracranially into three groups of CBA mice, respectively. The mice were killed at day 1, 3 and 7 p.i., and the organs removed as in the previous experiment.

The three heart-adapted viruses proved to be more infectious to myocardium than the original virus. In other words, they replicated to a higher titer in the heart than parental TMEV [F (1,39) = 11.25, p = 0.002]. Furthermore, the percentage of mice with evidence of infectious virus in the heart was also increased compared to parental TMEV, from 1/14 (7%) to 20/32 (62.5%) overall, as well as at each time point examined, as depicted in Figure 4-2A, B and Table 4-1. The virus was detected as early as day 1 p.i., in HAV-infected mice, while not seen until day 7 p.i. in the mice infected with parental TMEV.

The HAV propagated from 3 mice, noted as HAV-1, 2 and 3, also showed a diverse infectivity in myocardium (Table 4-2). HAV-3 caused heart infection as early as day 1 p.i. and this strain of virus was consistently detected at day 3 and 7 p.i., as well as with the highest percentage among the three HAV strains. The other two strains HAV-1 and 2 were first found at day 3 p.i., and were detectable in only 50% (2/4) of mice at day 7 p.i.

Table 4-1 The viral titers in the heart of non-restrained CBA mice infected with parental and heart-adapted TMEV

		Day 1 p.i.	Day 3 p.i.	Day 7 p.i.
Parental TMEV	Percentage of infected mice	0% (0/3)	0% (0/3)	33.3% (1/3)
	Average titers (log 10PFU/g)	0	0	3.13
Adapted TMEV	Percentage of infected mice	22.2% (2/9)	100% (9/9)	64.3% (9/14)
	Average titers (log 10PFU/g)	0.25	1.78	1.98

Heart-adapted TMEV includes 3 strains of virus recovered from three animals, respectively. Average titer refers to the mean titers of all the identified heart-infected mice, and those without detectable virus are not included.

Table 4-2 The comparison of viral titers in the heart of CBA mice infected with different strains of heart-adapted virus

		Day 1 p.i.	Day 3 p.i.	Day 7 p.i.
HAV-1	Percentage of infected mice	0% (0/3)	100% (3/3)	50% (2/4)
	Average titers (log 10PFU/g)	0	1.71	2.07
HAV-2	Percentage of infected mice	0% (0/3)	100% (3/3)	50% (2/4)
	Average titers (log 10PFU/g)	0	1.7	2.01
HAV-3	Percentage of infected mice	66.6% (2/3)	100% (3/3)	83.3% (5/6)
	Average titers (log 10PFU/g)	1.13	1.94	1.87

HAV-1, 2 and 3 denotes the three strains of virus recovered from the hearts of three infected/restrained mice infected with parental TMEV and sacrificed at day 7 post infection. Average titers are represented by the mean viral titers from the mice in which virus was detected in the heart.

This finding implied a variation in biological characteristics of HAVs, which may result from the stress process and result in the differences in viral clearance in the heart.

Because of the embryonic and histological homogeneity of skeletal muscle with myocardial muscle, we additionally removed the gastrocnemius muscle in order to determine if TMEV also displayed infectivity in this muscle. Interestingly, a very low replication of TMEV was seen in gastrocnemius muscle at day 7 p.i. in 6/14 mice, confirming the tropism of TMEV also towards skeletal muscle (data not shown).

The heart-adapted virus showed no difference in viral titers in the brain, spinal cord, spleen and thymus as compared to the parental TMEV strain (data not shown). To further verify that the heart-infected virus was Theiler's virus, rather than another potential cardiovirus, the virus was replicated in L2 cells and proved to be Theiler's virus by radioimmunoassay using anti-Theiler's antibody (data not shown).

3.3.2 The pathological alterations in the mice infected with heart-adapted TMEV

We observed no difference in the histopathological changes of the CNS and spleen between the HAV-infected and parental TMEV-infected animals. However, a focal, mild inflammation was observed in the heart of HAV infected mice (Figure 4-4E).

4. Discussion

In the present study, we investigated the effects of RST on TMEV infection in the CNS and systemic dissemination throughout the body. Furthermore, we also examined the change in pathogenecity of TMEV that develops following restraint stress.

Our data demonstrated the profound effect stress on the dissemination of TMEV.

The finding that higher titers of virus were detected in the brain and spinal cord of restrained mice suggests that stress impaired and/or delayed the viral clearance from the CNS. However, the delay was not evident until day 7 p.i., presumably because the virus replicated in both restrained and non-restrained mice during the first few days of infection in the absence of an effective immune response. However, at 7 days p.i. the immune response to TMEV takes effect and begins to reduce viral titers in the non-restrained mice. In the restrained mice, the delay in the development of an effective immune response, as evidenced by reduction of inflammatory infiltrates into the CNS of infected/restraint mice at day 7 p.i., allowing for continued viral replication. These findings temporarily correlated with the decreased chemokine expression at day 7 p.i. in the CNS resulting from RST (Mi et al., 2004).

Viral replication occurred in certain lymphoid organs and stressed mice generally developed higher viral titers in these tissues. The thymus did not appear to be a preferential tissue for replication of TMEV, since virus was not detected in the thymus of the non-restrained mice and the majority of the restrained mice (only 2 I/R mice had detectable virus in the thymus). In contrast, the spleen was greatly affected by RST, such that increased viral titers were noticed as early as day 3 p.i., which occurred earlier than the CNS; but the suppressive effect was attenuated with time, so that most of the restrained mice cleared the virus from the spleen by day 7 p.i. We have previously reported that restraint stress resulted in reduced chemokine expression in the spleen following at day 2 p.i., which resolved with time (Mi et al., 2004). Nonetheless, it might imply that the glucocorticoid resistance of splenocytes plays a role in restraint stress-induced immune responses, as seen in social disruption stress (Johnson et al., 2004; Stark

et al., 2001; Quan et al., 2003).

Morphologically, the thymus responded by a prominent reduction in size while the size of the spleen was moderately altered. These results could be explained, at least in part, by the fact that the thymus has greater glucocorticoid receptor expression than the spleen (Miller et al., 1998), which would result in a greater degree of apoptosis induced by glucocorticoids in the thymus (Ahmed and Sriranganathan, 1994; Goya et al., 2003). Additionally, thymocytes are primarily immature T cells ($CD4^+$ $CD8^+$ T cells), which are more sensitive to glucocorticoid induced by stress (Tarcic et al., 1998); whereas the spleen contains fewer immature T cells when compared to the thymus.

Generally, RST tends to increase viral titers in cervical LN over the seven days of infection, despite the fact that comparable virus loads were detected in the cervical LNs of both the restrained and non-restrained mice from the individual time point examined; while TMEV was found primarily in the mesenteric LNs and lumbar LNs of the restrained mice at later times (day 3 and 7 p.i.). The differential effect of restraint on the viral clearance among cervical LNs, mesenteric LNs and lumbar LNs may be associated with the route of infection. Since the virus was first introduced into the brain, TMEV in the parenchyma and meninges would drain to the cervical LNs (Bradbury et al., 1981), possibly for priming T cells (Weller et al., 1997). The detection of virus in the lumbar and mesenteric LNs at a later time may suggest the dissemination of TMEV from the brain to the spinal cord and abdominal organs following stress, which resulted in infection of these organs and drainage by lumbar and mesenteric LNs.

RST had little effect on the morphology of LNs. The underlying mechanisms of stress on the LNs were not investigated in this study. It has shown that glucocorticoids

influence tissue-specific endothelial cells to interact with immune cells (Chung et al., 1986) and the expression of lymphocyte adhesion molecules for the entry into LNs, such as L selectin (Sackstein and Borenstein, 1995), thereby inhibiting the homing of lymphocytes to LNs and subsequent effects on local immunity.

In addition to the CNS and lymphoid organs, the fact that the widespread dissemination of TMEV occurs following stress demonstrates that repeated restraint stress increases the susceptibility to systemic viral infection.

Interestingly, we discovered focal hepatic coagulative necrosis exclusively in stressed mice, regardless of infection status. The pathogenic mechanisms responsible for this tissue injury remain unclear**.** It was reported that stress, trauma and sleep deprivation may enhance translocation of gastrointestinal bacteria to the liver and spleen (Ando et al., 2000; Ding et al., 2004; Everson and Toth, 2000; Wang et al., 2004). However, we were unable to detect bacteria in the liver of restrained mice using Gram staining and therefore other mechanisms may account for the liver lesions. We speculate that the elevation of circulating glucocorticoids or tumor-necrosis factor (TNF-α), as detected in previous studies (Welsh et al., 2004) could result in hepatotoxicity. Additionally, stress-induced immunosuppression may impair the barrier which the liver serves for preventing the entry of intestinal endotoxin, consequently causing overburden of the liver function. In support of this hypothesis a recent study demonstrated that stress interferes with hepatic lipid peroxidation resulting an increase of γ-glutamil transferase (γ-GT) (Correa et al., 2004), a marker for liver injury.

In terms of infectivity, our data showed that restraint significantly increased parental TMEV replication without inducing remarkable lesions in the myocardium. The absence

of lesions may be due to a short time after infection at which the tissues were examined, decreased viral virulence and/or different strains of mice used in this study. Roos et al. (1996) found that various degrees of myocardial lesions were detected in different strains of mice when intraperitoneally infected with DA virus. Supporting evidence showed that a conspicuous lesion and higher viral load in the myocardium were observed when mice were infected with *more virulent* GDVII or DA derivatives compared to original DA and BeAn strains (Rames et al., 1994; Rames, 1995).

In the final experiment of the current study, heart-adapted virus isolated from stressed mice, when injected via the same route, with the same dosage but without restraint, produced an altered pathogenecity that was manifested by an early (day 1 p.i.) and higher myocardial infectivity, as compared to those animals infected with the parental TMEV. This alteration in infectivity was accompanied by a mild inflammation in the myocardium.

The reason for the altered pathogenecity was not examined in the current study, but it is believed that multiple factors may contribute to this change. First, the higher level of virus in stressed mice allows the generation of increased numbers of mutant viruses with altered pathogenecity. For example, TMEV may be readily mutated on VP1 and VP2 loops of capsid, resulting in alteration in sialic acid (the receptor for entry of persistent Theiler's virus) binding ability and virulence (Jnaoui et al., 2002; McCright et al., 1999). Second, the endocytosis of TMEV is thought to be a receptor-mediated process. It was shown previously that the sialic acid binding receptors on rat macrophage (Gessl et al., 1989) and sialic acid in HeLa cells (Sinha and Melnykovych, 1972) may be up-regulated in response to glucocorticoids. Thus, the increase in sialic acid expression on the cell

surface may facilitate the entry of virus into the cells, and account for the increased infectivity following the exposure to elevated glucocorticoids. Third, Huber et al. (1990) discovered that viral receptors expressed in vascular endothelial cells of different organs vary in binding activity for the virus. They documented that Coxackievirus isolated from a certain organ of systemically infected animals exhibited preferential infectivity for the same type of organ, i.e. the virus recovered from the heart proved to be more infectious to the heart than any other organ, suggesting the crucial role of VEC in mediating the virus tropism. These findings could explain that the infectivity in the CNS by either parental or adapted TMEV showed no difference.

Our results provide evidence for various possible pathways for the dissemination of TMEV following intracerebral inoculation. The nature of progression of viral replication in the spinal cord suggests that the dissemination of virus from the inoculated brain to the adjacent spinal cord. This process may help explain the development of TMEV-induced disease in which the virus is cleared from the brain and persists in the spinal cord during the chronic phase. Even though the viral load of both tissues were very similar at day 7 p.i., less severe inflammatory lesions were observed in the spinal cord than in the brain, which suggest that the severity of lesion may correlate with not only the viral load, but also the duration of exposure to a certain viral load. The mode of TMEV dissemination from the brain to the spinal cord remains uncertain; however, it is most likely via direct spread of viral progeny to neighboring neurons (Ha-Lee et al., 1995), and/or astrocytes (Aubert and Brahic, 1995) or by via axonal and dendritic transportation (Dal Canto and Lipton, 1982; Rodriguez et al., 1983; Stroop et al., 1981).

The detection of virus in such vascularized organs as the spleen and the liver,

indicate that the hematogenous spread could be another route for the dissemination of TMEV which may occur immediately following the intracerebral infection. It has shown that the TMEV can infect and replicate in cerebrovascular endothelial cells (Welsh et al., 1995; Zurbriggen and Fujinami, 1988), supporting this as a possible mechanism for entry into the circulation. In addition, the hematogenous spread of TMEV has been confirmed by the isolation of the virus from blood (Borrow, 1989). Such a viremia may be responsible for systemic infection of other organs that include the lungs, heart and gastrointestinal tract, as shown in our data.

Another potential pathway for viral dissemination is via the lymphatic circulation as evidenced by detection of the virus in the lymph nodes. Viral infection of cervical LNs was observed as early as day 1 p.i. (10^4 PFU/g) suggesting that early infection of inflammatory cells from the brain were drained to the local LNs and thereby circulated throughout the body via the lymphatic system. Although the specific manner by which virus spreads from and to LNs could not be determined, macrophages have been implicated to be a reservoir for TMEV in the late stage of TVID (Clatch et al., 1990; Lipton et al., 1995; Rossi et al., 1997). In spite of these possible pathways of virus spread, the dissemination of TMEV may also vary depending on the route of infection (Ha-Lee et al., 1995; Gomez et al., 1996).

In the present studies, we report that the repeated restraint stress profoundly impacts the viral clearance from the CNS and systemic organs during TMEV infection, and alters the pathogenicity of this virus. These profound effects, including the liver injury induced by restraint, may explain the exacerbated clinical symptoms and higher mortality in stressed and infected animals, which are not common in non-stressed animals. However,

the viral distribution within the diverse tissues and the mechanisms by which the virus enters these cells require further investigation. The fact that stress alters the pathogenesis of TMEV infection may have important implications for other disease processes. Stress may render a virus pathogenic for diverse organs and result in novel diseases.

V THE EFFECT OF DURATION OF RESTRAINT STRESS ON THE LATE DEMYELINATING DISEASE

1. Introduction

Multiple sclerosis (MS) is the most common demyelinating disease of the central nervous system (CNS) in humans with a higher incidence in females than in males. The causative agents of the MS are unclear; however, several environmental agents, such as viruses and stress, have been suggested in the pathogenesis of MS. The disease is characterized by multiple plaques found in the white matter of the CNS, accompanied by inflammatory infiltrates in the lesions, an indication of immune-mediated involvement.

Psychological stress has been reported to influence the clinical course of MS, in which the stressors may result in exacerbation of MS symptoms (Ackerman et al., 2002; Mohr et al., 2004; Strenge et al., 2001). It appears that the association between stress and aggravation of MS is not always consistent in that certain stressors may ameliorate the disease. This suggests that the impact of stress on MS may depend upon the type and intensity of the stressor (Mohr and Cox, 2001; Nisipeanu and Korczyn; 1993). The underlying mechanism of the effects of different stressors on MS remains unclear. The dual role of stress on disease course has been extensively studied in animal models for MS, such as Experimental Allergic Encephalitis (EAE).

EAE is induced by the injection of myelin components or CNS homogenates in complete Freund's adjuvant into mice or rats to produce demyelination. If the animals are then subjected to repeated or prolonged restraint stress prior to challenge of myelin basic protein (MBP), it has been demonstrated that a decreased incidence and severity of the

disease in rats and mice (Levine et al., 1962; Whitacre et al., 1998). Whitacre et al. (1998) also reported that one hour of restraint per day exacerbated the clinical symptoms of EAE, rather than suppression as observed by nine hours of restraint stress. Acute pre-stressed mice displayed an early onset of EAE (Chandler et al., 2002). The bidirectional regulation of stress was also documented in experimental arthritis, in which chronic intermittent noise suppressed the severity of bradykinin-induced adjuvant-arthritis (Strausbaugh et al., 2003), whereas a one-hour noise stress per day prior to type II collagen immunization aggravated arthritis (Rogers et al., 1983).

The differential immune and neuroendocrine responses following various stress exposures have been studied and well-defined by Dhabhar and McEwen (1997). They proposed a bidirectional effect on the immune system by a differential type of stressor, which is based on the duration, intensity of stressor and perception of the stress by the subject. An acute stressor, which is short-term lasting for minutes to hours, allows for the adjustment and adaptation of the organism to a changed situation, known as "eustress" and results in immune-enhancement; whereas chronic stressor, which is long-term and lasts for days to months, ultimately leads to maladaptive and pathological processes, know as distress and is thought to be immunosuppressive. They also demonstrated an enhanced skin delayed type hypersensitivity (DTH) following acute stress and a reduced cutaneous DTH in response to chronic stress. The enhancement of DTH response following acute stress was due to an alteration in leukocyte trafficking to the skin (Bilbo et al., 2002; Dhabhar and McEwen, 1997). Other authors have reported that an acute stress increased the immunoglobulin (Ig) response following immunization of sheep red blood cell, while a prolonged stress suppressed the antibody response (Millan et al., 1996;

Silberman et al., 2003; Zalcman and Anisman, 1993). Furthermore, mitogen-induced T cell proliferation was increased by acute stress (Bauer et al., 2001) but decreased by chronic stress (Silberman et al., 2002).

It appears that the duration of stress is an important index in defining acute and chronic stress. It is well established that activation of the hypothalamus-pituitary-adrenal (HPA) axis and sympathetic nervous system (SNS) mediates the stress-induced immune response through the release of glucocorticoids (GC), epinephrine and norepinephrine from the two axes. Other stress hormones, such as corticotropin releasing hormones (CRH), adrenocorticotropic hormone (ACTH), opiates and prolactin, are secreted in a sequential, ordered pattern in response to stress. Each hormone has its own kinetics, thus indicating that differential effects of acute and chronic stress may reflect a temporally combined effect of all the stress hormones (Sapolsky et al., 2000). In addition, GC display a dual action: a modulating action, to produce a stress response; and a preparative action, to set up a response to the subsequent stressor and develop an adaptation (for review, see Sapolsky et al., 2000). Furthermore, the role in pharmacological manipulation of GC for immunosuppressive therapy differs from the physiological function of endogenous GC, which suggests the complexity of the function of GC. It is also proposed that an animal mounts a rapid response (immunostimulating) to an acute stressor; however, if this response is not shut-off in a timely manner, for example, following exposure to a chronic stressor, a high "allostatic load" will eventually "wear and tear" the system, resulting in immunosuppression (Dhabhar, 2003). The underlying mechanisms mediating the acute and chronic stress remain to be elucidated.

Epidemiological data revealed a gender effect in MS, in which MS affects females

more frequently than males. It is conceivable that sex hormones such as estrogen and progesterone may play a role in this disease. Pregnancy, especially in the third trimester, appears to be associated with a lower disease rate and less severe relapses. Such a protective effect on MS course during pregnancy is thought to be related to high estrogen levels; whereas the exacerbation of MS is observed during postpartum and the menstrual cycle when estrogen levels are low (Pozzilli et al., 1999; Salemi et al., 2004). The gender difference was also documented in the mouse EAE model, in which females exhibit a greater susceptibility to CNS lesions (Teuscher et al., 2004). The therapeutic effect of estrogen through estrogen receptors in male EAE (Palaszynski et al., 2004a), as well as the protective role of androgens in EAE (Palaszynski et al., 2004b) implies the relationship between sex hormones and the immune system. However, the gender effect in the induction of demyelination is dependent on both the antigens used for immunization and the genetics of mouse (Okuda et al., 2002). The association of a stress and a gender effect in EAE has been reported previously. For example, female rats are protected from the acute and stress-induced exacerbation of EAE, which was not seen in male rats; the disease was further suppressed in female rats than males following chronic stress (Whitacre et al., 1998).

The present series of experiments were designed to investigate the role of acute or chronic stress on the pathogenesis of MS using a Theiler's virus induced demyelination model for MS. Intracerebral inoculation of Theiler's virus in susceptible strain of mice causes an acute encephalitis during the first two week p.i., and a chronic progressive demyelination detected about four weeks p.i. (Lipton, 1975). The persistent viral infection in the CNS is critical for the development of demyelination, which is mediated

by myelin-specific autoreactive CD4+ T cells via epitope spreading (Miller et al., 1997; Welsh et al., 1989). We have previously shown that a chronic restraint stress markedly suppresses natural killer cell activity (Welsh et al., 2004), chemokine (Mi et al., 2004) and specifically pro-inflammatory cytokine expression (Mi et al., manuscript in preparation), resulting in impaired viral clearance in the CNS (Mi et al., submitted) and exacerbation of subsequent demyelination (Sieve et al., 2004). Due to the potential immunoenhancing role of acute stress, we hypothesize that the exposure of mice to a 2 hour session of acute restraint prior to the viral inoculation will augment the antiviral immunity in the early infection, thus causing an enhanced viral clearance. Consequently following acute restraint stress, there should be an improvement in the late demyelination as compared to either non-restrained or chronically restrained mice. Additionally, the effects of stress were examined in both female and male mice, so as to determine the effects of both gender and stress in Theiler's virus-induced demyelination (TVID).

2. Materials and methods

2.1 Subjects

SJL/J mice were bred in the animal facility at Texas A&M University using breeding pairs obtained from Charles River laboratory (Boston, Massachusetts). Mice were maintained under a pathogen-free environment in a 12:12 light/dark cycle (lights on from 6:00 am to 6:00 pm). At three weeks of age, male pups and female pups were housed in separate rooms. Mice were allowed to acclimate to such conditions for one week prior to any experimental manipulations.

2.2 Infection

The BeAn strain of TMEV (kindly provided by Dr. H. L. Lipton, Department of Neurology, University of Chicago, Evanston, IL) was propagated and titrated on L2 cells by a standard plaque assay and stored at -80^{o}C (Welsh et al., 1987). $7x10^{4}$ PFU BeAn virus in 20µl inoculum was injected intracranially into the right cerebral hemisphere of mice at 4-weeks of age. Mice in non-infected groups were sham-infected with 20µl sterile phosphate-buffered saline (PBS).

2.3 Restraint stress paradigm

Mice were physically restrained in a 60 ml polypropylene syringe with perforated ventilation holes of 0.4 cm diameter. Mice were either acutely restrained for a single session of 2 h (5am-7am) or chronically restrained for 8 h (12:00 am-8:00 am), for five consecutive nights per week, with two nights off in between weeks, for a total duration of four weeks. The viral inoculation occurred 15 minutes following release from the restraint tube for the acute restrained mice or 2 h after the first session of chronic restraint for the chronically stressed mice (Campbell et al., 2001; Dhabhar and McEwen, 1997; Sheridan et al., 1991).

2.4 Clinical scoring

Clinical symptoms were evaluated according to a numerical score for signs of demyelination and paralysis as described previously (Borrow et al., 1998; Sieve et al., 2004): 0 = no behavioral impairment, 1 = weakness in hind limbs, 2 = slightly wobbly gait, 3 = definitely wobbly gait, 4 = very wobbly gait, hunched posture, and loss of

righting reflex, 5 = all of the signs mentioned above, and incontinence, 6 = moribund.

2.5 Footprint analysis

Footprint stride and spread has been reported as a sensitive indicator of neurological deficits caused by demyelination or axonal loss in mice (Johnson et al., 2004; McGavern et al., 1999; McGavern et al., 2000). Briefly, the forelimb and hindlimb paws of mice were painted with red and blue nontoxic paint and the mice walked along a 2.5 by 3.6 inch runway lined with white paper. A minimum of eight forelimb and eight hindlimb paw prints per mouse were used for the three spreads and three strides measurement.

2.6 Corticosterone assay

Mice were bled from the saphaneous vein between 7:30am- 9:30 am, immediately after releasing from the restraint tubes at various times throughout the course of the experiment. The bleeding procedure has been described elsewhere (Johnson et al., 2004, Sieve et al., 2004). Plasma samples were obtained by centrifugation and separation, and then were stored at -80^0C until assayed. Plasma corticosterone (CORT) level was determined by radioimmunoassay (RIA) using a ^{125}I-RIA kit (ICN Biomedicals, Costa Mesa, CA).

2.7 The determination of antibody to Theiler's virus and myelin proteins

The plasma antibodies against Theiler's virus, myelin basic protein (MBP), myelin oligodendrocyte glycoprotein peptide (MOG_{33-35}) and proteolipid protein peptide ($PLP_{139-151}$) were measured using a RIA, as described previously (Dolimbek et al., 2002; Sieve et

al., 2004; Young et al., 1983). The assay was developed using radiolabeled protein-A which binds to the Fc portion of immunoglobulin.

Briefly, a flexible u-shaped, 96-well polyvinyl chloride plate (Costar, Cambridge, MA) was initially washed with PBS/Tween-20 (0.05% v/v) and water purified by reverse osmosis (RO H_2O). The plate was coated with 100 µl of carbonate buffer (pH 9.6) containing Theiler's virus (1.0×10^7 p.f.u./100 µl) for anti-TMEV antibody determination. For assessment of MBP, MOG and PLP peptides, 100 µl assay buffer (made up from two parts: 495 ml of part A: 0.08M Trizma HCl, 0.03M Trizma base and 0.15M NaCl at a final pH of 7.2, and 5 ml of part B: 1.0% NFDM and 0.5% Tween-20 in RO H_2O) containing 1.0 µg of either MBP (from bovine; Sigma, Saint Louis, MO 63103 USA), MOG $_{33\text{-}55}$ (Sigma), or PLP $_{139\text{-}151}$ (AnaSpec, California) was added to the wells. The plates were incubated at 4°C for 24 hours and then washed as described above. The plate was blocked with 3.0% non-fat dry milk (NFDM) in PBS (pH 9.0) for one hour at 37°C. After washing, either a positive control rabbit serum (generated by 3 serial injections of UV inactivated BeAn strain of Theiler's virus into a rabbit), or goat polyclonal IgG anti-MBP and goat polyclonal IgG anti-MOG antiserum (Santa Cruz Biotechnology, California) or an anti-goat negative control serum from rabbit (Sigma, Saint Louis Missouri, USA) were diluted 1/20 in assay buffer and added to the wells (100 µL total volume). Doubling dilutions of antiserum were carried out until a final dilution of 1/640 was achieved.

Following the serial dilutions, the plate was then incubated for one hour at 37°C before washing the plate and rinsing again (as previously described). Then, a polyclonal goat anti-rabbit (Sigma, Saint Louis, Missouri, USA) antibody was diluted 1/500 in assay

buffer, and 100 µl was added to every well. After incubating at 37°C for one hour and a subsequent washing, 100 µl of ^{125}I-Protein A (1 x 10^5 cpm/100 µl assay buffer) was added to each well, and the plate was incubated at room temperature for one hour. The plate was then washed and rinsed with PBS/Tween-20 and RO water (as described above). Once the plate was dry, every well was cut out, put into tubes and counts were determined by using a gamma counter (Beckman gamma 5500 counting system).

2.8 Histological examination

Mice were sacrificed by the i.p. injection of pentobarbital at day 220 p.i. and perfused with PBS into the right ventricle of the heart. Left or right hemisphere of the brain, and coronal sections of the spinal cord were alternatively collected for either viral titration or microscopic examination. The thymus, spleen, heart, liver, adrenals, sciatic nerve and gastrocnemius muscle were aseptically removed for histological analysis.

The tissues for histological examination were fixed in 10% formalin, embedded, sectioned and stained with hematoxylin & eosin (H&E) for microscopic examination of lesions. For further evaluation of demyelination and inflammation, spinal cords were stained with Weil's myelin staining and Holmes staining respectively. B cells, T cells, macrophages and astrocytes were stained for analysis of the cellular phenotype during demyelination using an immunochemistry.

The loss myelin sheath was demonstrated by Weil's staining. Briefly, a 10-15µm paraffin section of spinal cord was deparaffinized and immersed in 5% celloidin for immobilization of the section on slides. After washing the section with distilled water, the stained with 4% iron aluminum (ferric ammonium sulfate, $FeNH_4(SO_4)_2$-$12H_2O$) and 1%

hematoxylin at 50-60^{0}C, for 40 min, followed by rinses with tap water. Then the section was placed in 4% iron aluminum until the gray matter or degenerated area can be differentiated and was washed well in several changes of water. For complete differentiation, the section was stained with Weigert's borax ferricyanide solution [2g sodium borate ($Na_2B_4O_7$-$10H_2O$) and 2.5 g potassium ferricyanide ($K_3Fe(CNO_6)$ in 200 ml distilled water] for 3 min, followed by washes with distilled water and tap water. Next, the slide was placed in 0.5% lithium carbonate for 1 min. After several rinses with water and dehydrated, the slides was ready for microscopic examination.

2.9 Determination of viral titers

The remaining brain and spinal cord were weighed and homogenized in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen Corporation, USA). Cellular debris was removed by centrifugation and the titer of virus in the supernatant was determined by standard plaque assays on L2 cells.

2.10 Experimental design

A 2 (infected vs. non-infected) by 3 (acute restraint, chronic restraint and non-restraint) by 2 (male vs. female) factorial design, 5-15 mice per cell, was applied in this study. Baseline footprint and plasma corticosterone were measured. To monitor the alteration in clinical manifestation following TMEV infection and restraint stress, clinical scoring was assessed weekly, and footprint measurement was performed every 10 days in the first month postinfection, twice in the second month and monthly thereafter during chronic disease. Mice were sacrificed at day 220 p.i. when most infected animals

developed clinically marked paralysis.

2.11 Statistical analysis

Analyses of variance (ANOVA) were conducted on the data for the three variables restraint, infection and gender. Duncan's multiple range tests and means comparisons were used for post hoc analyses. A p value of 0.05 or less was considered significant in all cases.

3. Results

3.1 Clinical scoring of signs of neurological impairment

As depicted in Figure 5-1, the main effects of infection, RST and day p.i. on clinical signs were observed, all Fs > 15.714, ps < 0.0001. There were also interactions of infection and stress, day post infection and infection, day post infection and stress, as well as interactions among day post infection, infection and stress, all Fs > 4.36, ps < 0.0001, wherein restraint stress exacerbated clinical symptoms in infected mice; furthermore, the degree of exacerbation was increased over time. Clinical symptoms appeared earlier in chronically stressed mice as compared to non-stressed animals; whereas the onset was delayed in the 2-hours acutely stressed mice. However, the progression in severity of clinical sign appeared to be converging during the later stage of disease. No gender difference in clinical signs by stress was observed. Additionally, there was a progressive, wavelike trend in the clinical signs, particularly in the infected/non-restrained mice, which may suggest a relapsing-remitting disease process.

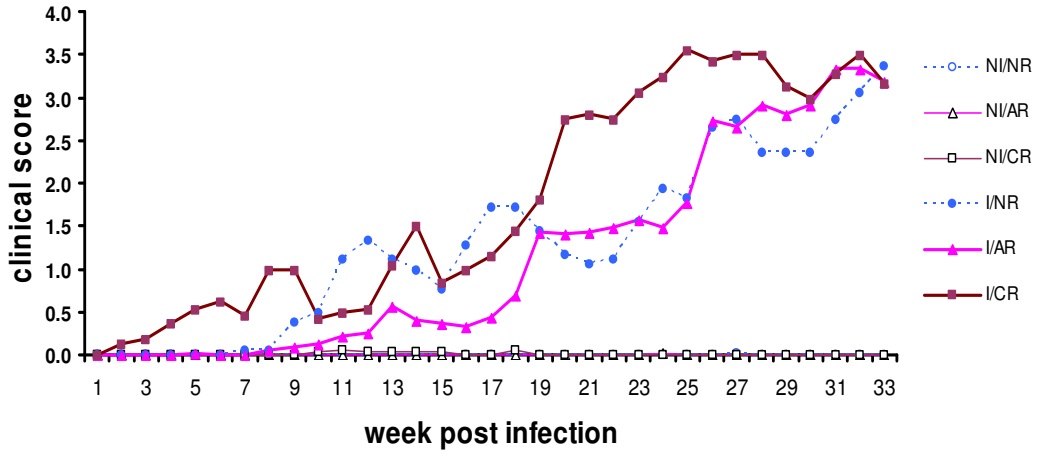

Figure 5-1. Clinical score following infection and various duration of restraint stress during the course of TVID.

3.2 Stride length

As shown in Figure 5-2, using baseline as a covariate, there was a main effect of infection, (F = 29.29, p = 0.0001) and restraint stress (F = 6.822, p = 0.0023) on stride length. The interaction of day p.i. by infection on stride length was qualified by ANOVA (F = 3.973, p = 0.002), such that the stride length was reduced with time in infected mice.

The effects of the variables, infection, restraint and gender on stride length at individual time points by ANOVA showed that a reduction in stride length in all infected, as compared to non-infected, mice was first detected at day 45 p.i., F (1, 50) = 4.735, p = 0.03, and that this reduction was not further observed until at day 120 p.i. and remained up to day 220 p.i. at the time of sacrifice, all Fs (1, 39) > 3.965, ps < 0.05. A main effect of stress was found at day 20 p.i., wherein chronic stressed mice, independent of infection, displayed a decreased stride length, F (2,46) = 17.339, p = 0.0001. This may be due to that, at day 20 p.i., the mice were in the last session of chronic stress period, which started from day –1 p.i. up to day 24 p.i., suggesting that long and repeated stress may impact motor function. However, this effect may disappear with the termination of stress session, so that the stress effect was not observed at day 30 p.i., which was the sixth day after the end of stress. At day 45 p.i., all stressed mice showed a decreased stride length as compared to the nonstressed mice, F (2,50) = 11.548, p = 0.0001.

ANOVA also revealed an infection by restraint stress interaction on stride at day 195 and 220 p.i., all $\underline{F}$s > 4.322, $\underline{p}s$ < 0.04. Post hoc analysis confirmed the varying effect of acute and chronic restraint stress on stride length in infected animals, such that a descending order in hind limb stride length was seen in non-restrained, acute restrained and chronic restrained mice, respectively, indicating an impact of various duration of

restraint stress on the impairment of motor function. This trend was also noted as early as day 30 and 60 p.i., but failed to reach significance. No gender effect and other interactions of these variables were identified.

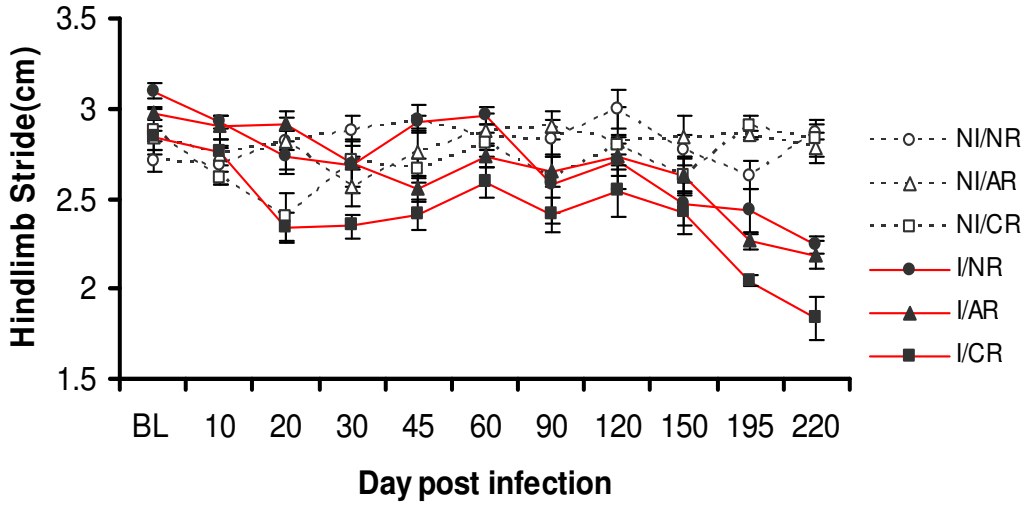

Figure 5-2. Footprint of hind limb stride length following infection and restraint stress. The data was derived from 8 footprints per mouse, and are represented by mean $\pm$ S.E.M. n=10-16 mice for each experimental condition. BL: baseline, was measured 2-3 days prior to infection or restraint was applied.

3.3 Plasma CORT levels

A "main effect" of infection and stress on the baseline level of plasma CORT collected from the mice at 3 days prior to infection. This could be a sample error, since neither infection nor stress was applied to the animals at the time of bleeding. The plasma CORT after the first session, either acute (2 h) or chronic stress (8 h), and before infection, was assayed to examine the impact of the various restraint stress sessions on its secretion. ANOVA showed a main effect of stress on CORT level, wherein 8 hr restraint resulted in significantly higher plasma CORT (13-fold increase) than 2 h restraint (2.5-fold increase) (Figure 5-3A). As depicted in Figure 5-3B, a main effect of gender was found on CORT level, such that females always produce a higher CORT than males (F = 6.916, p = 0.015), but the difference was not significant at baseline, F (1,16) = 2.753, p = 0.1. An interaction of restraint by gender revealed by ANOVA suggested that females had a higher CORT response to restraint than males, all Fs (1,16) >6.816, ps < 0.019 (Figure 5-3B). In addition, the CORT level was also measured at day 7 p.i. (acute restraint had ended 7 days previously), and it declined to the baseline level as compared to controls (F = 2.651, p = 0.11) (data not shown). No effect of infection on CORT was found.

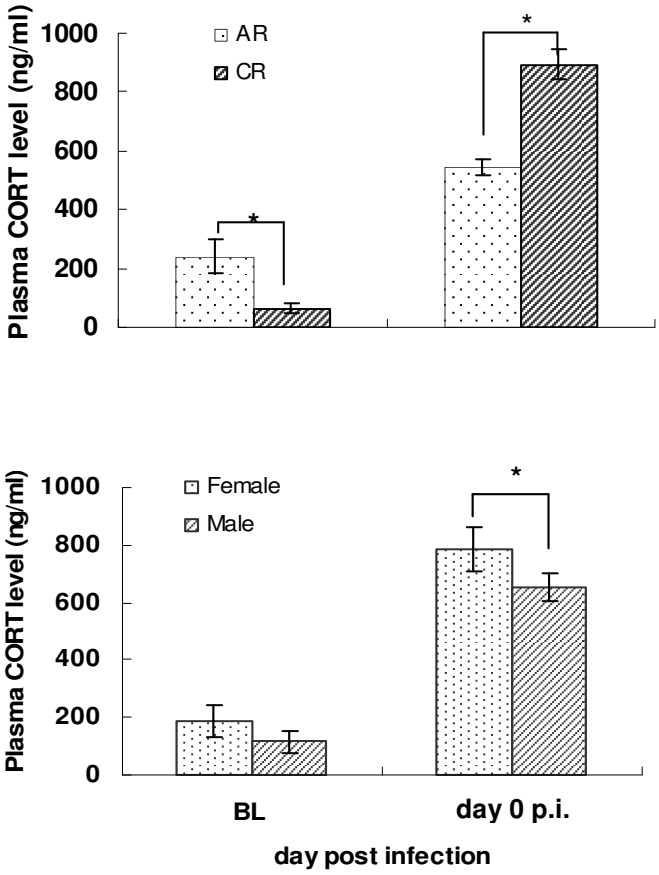

Figure 5-3. The effects of various duration of restraint stress and gender on plasma CORT response. (**A**) Plasma CORT level in response to acute (2 h) or chronic restrained (8h) in female and male mice (**B**). BL, baseline CORT was measured 3 days prior restraint was applied. The blood was sampled after the 2 h restraint or the first 8 h session and the level was determined using RIA assay. * indicated $p<0.01$.

3.4 Antibody measurement

In all the infected groups (I/NR, I/AR, I/CR), there was a main effect of stress on anti-TMEV antibody production, such that a higher antibody level was detected in non-restrained than either acute or chronic restrained mice. The differences in antibody production were observed at days 28 and 220 p.i., all Fs > 3.438, ps < 0.046. Post hoc analysis found no difference in antibody level between acutely and chronically restrained mice throughout the course of disease (Figure 5-4). Considering that the bleeding procedure might be a further stressor to the chronic restrained mice, the I/CR mice were bled biweekly, rather than weekly during the first month post infection, so that the data at day 7 and day 21 p.i. included only I/NR and I/AR mice. ANOVA confirmed the same effect of stress in that the 2 h restraint suppressed the anti-TMEV production, all Fs > 7.136, ps < 0.032 (data not shown). No gender effect was seen in the TMEV antibody response.

Terminal bleeds were assayed for antibody against myelin components. A very low level of antibodies against MBP, MOG_{33-35} or $PLP_{139-151}$ peptides was detected at day 220 p.i. in any groups of animals and no effect of stress was found, all ps > 0.05. However, a main effect of gender on MBP antibody production was revealed by ANOVA such that females had a higher MBP antibody than males, F (1,28) = 7.393, p = 0.011. Furthermore, a decrease of MBP antibody was noticed in female chronic restrained (I/CR) mice as compared to non-restrained (I/NR) using Duncan's new multiple range test, p < 0.05 (data not shown). Very interestingly, a main effect of infection on $PLP_{139-151}$ antibody was discovered with a surprisingly elevated $PLP_{139-151}$ antibody in non-infected mice [F (91,46) = 7.013, p = 0.011], which was markedly increased in chronically restrained

(NI/CR) mice. No other effects on the MBP, MOG_{33-35} or $PLP_{139-151}$ antibody response was observed.

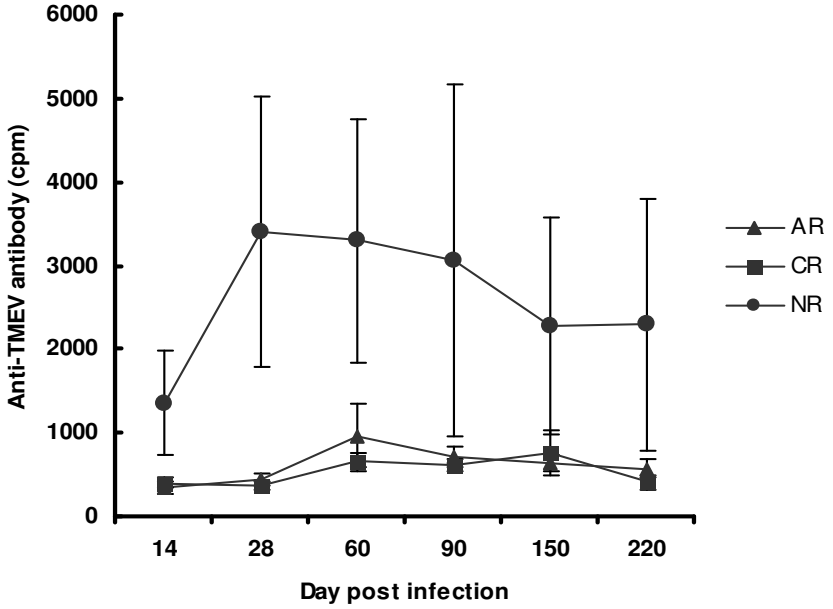

Figure 5-4. Anti-TMEV antibody response during the course of TVID. The data were derived from 10-15 mice per experimental condition. The values were 1/40 dilution and represented by mean $\pm$ S.E.M.

3.5. Microscopic examination

The spinal cord lesions were first examined by H&E staining. As seen in Table 5-1, there was a higher incidence of demyelination in chronically restrained mice (37.5%) than acutely restrained (20%) or non-restrained mice (12.5%). The lesions primarily involved the white matter in the thoracic spinal cord, and cervical spinal cord to a lesser extent, but rarely detected in the lower lumbar section. As shown in Figure 5-5 A, B, C, demyelination occurred mainly in lateral column, ventral lateral column and ventral area; however, dorsal area (gracile fasciculus and cuneate fasciculus) was not involved. The chronic restrained mice demonstrated the maximum percentage of demyelination, followed by acutely restrained, and the non-restrained mice displayed the minimum percentage of demyelination among the three groups (Table 5-1) The lesion in non-restrained and acutely restrained mice is characterized by loss of myelin sheaths in the white matter of spinal cord, as revealed by Weil's staining (Figure 5-5B). A prominent inflammation manifested by meningitis, cuffs, and increased inflammatory cellularity in the demyelinating areas (Figure 5-5A). Axons in these areas are usually preserved. The chronically restrained mice exhibited minimal inflammation, accompanied by axonal loss in some animals (Figure 5-5D and E), suggesting more severe myelin damage by chronic restraint; whereas the lack of inflammatory cells in these mice may indicate a later stage of demyelination observed at sacrifice in this study. (NOTE: more slides of Holmes stain and immunochemistry for cell phenotype in demyelination are pending).

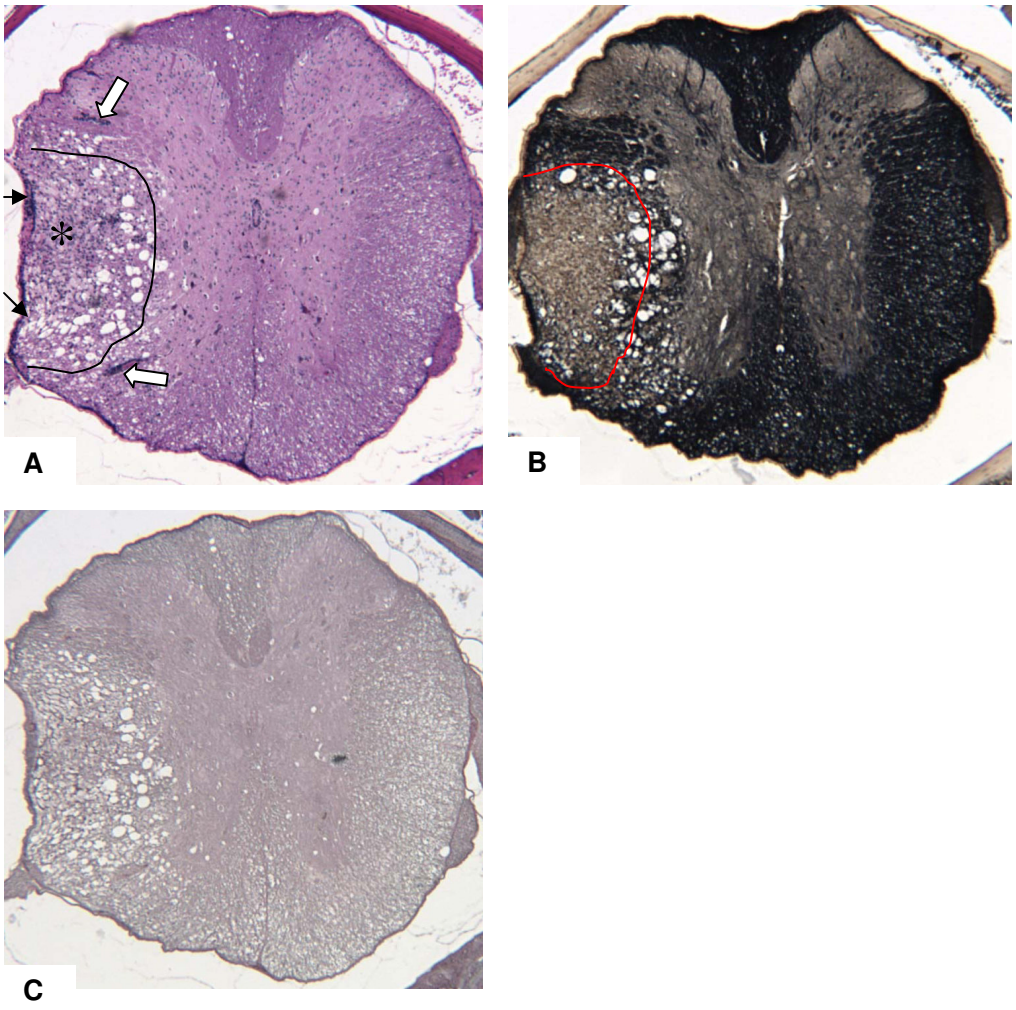

Figure 5-5. Histopathology of demyelination in the spinal cord of SJL/J mice at the late TVID (day 220 p.i.). **(A)** A representative section by H&E staining showed demyelination (marked by black line), meningitis (black arrow), perivascular cuffings (opened arrow), and inflammation in the demyelination area (*) (4X). **(B)** A serial section from the same mouse above confirmed demyelination, as indicated in the red line marked area, using Weil's myelin staining (4X). The demyelinated area should stain black as is illustrated in the normal white matter in the opposite side of the section. **(C)** A serial section of spinal cord by Holmes staining showing preserved axon (4X).

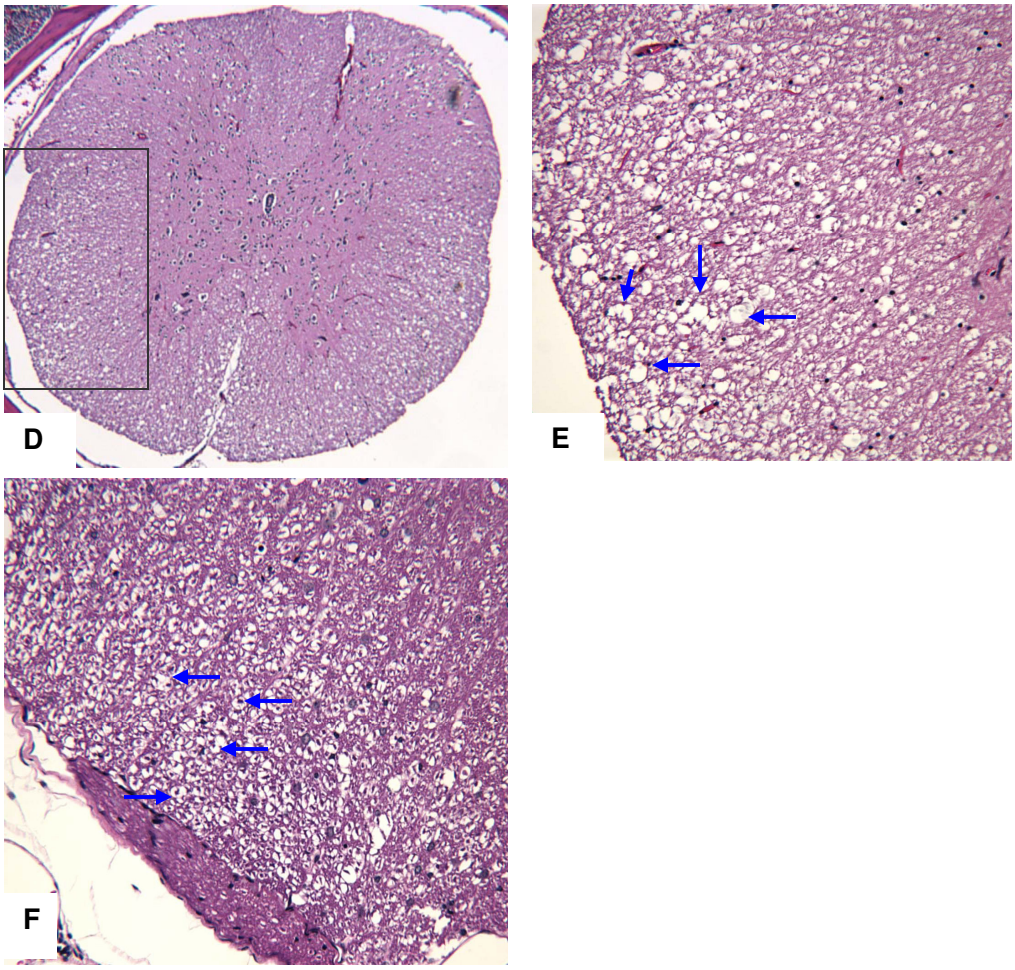

Figure 5-5. Continued. **(D)** A representative section of spinal cord of a chronically restrained mouse displays axonal loss, as indicated in the boxed area, but with few inflammatory infiltrates (4X). **(E)** A magnification (20X) showed loss of axon (blue arrow) in the spinal cord. **(F)** A section from the same mouse as E illustrated showing intact axon (blue arrow) (20X).

Table 5-1 Histological assessment of spinal cord during chronic demyelination

	Non-restrained	Acute restrained	Chronic restrained
Incidence of demyelination	12.5% (1/8)	20% (4/20)	37.5% (3/8)
Percentage of demyelination	17.70%	27.30%	46.50%
Axonal loss	(-)	(-)	(+)

Note: percentage of demyelination was caculated by the ratio of the area of demyelination against the area of white matter in the same section. The value was derived from the average of the sections in which demyelination was observed.

4. Discussion

The present investigation was designed to study the impact of the duration of stress on demyelination in Theiler's virus infection in mice as an animal model for human MS. We showed that RST applied during early infection appeared to exacerbate the clinical neurological impairment in the chronic disease, as indicated by clinical scores, walking stride length and incidence of demyelination. Repeat chronic restraint (4 weeks of 8 h session) exhibited the most profound impact on the clinical signs, which replicated our previous findings (Sieve et al., 2004); whereas 2 h acute restraint displayed an intermediate effect in worsening the symptoms.

The influence of stress on MS has been studied in another model for MS, namely the EAE model. It has been reported that the disease course may be altered by various stressors, and the outcome of disease is determined by the timing of the stress exposure. For example, restraint stress prior to immunization delayed the onset and severity of EAE in B10.PL mice (Dowdell et al., 1999), and in Lewis rats (Griffin et al., 1993). A combination of various stressors (swim, odor, crowding, restraint stress, etc) in rats prior to EAE induction attenuated the disease, but no such a protection was seen if given after the immune induction of disease (Correa et al., 1998).

In EAE, it has been shown that chronic pre-stress resulted in inhibition of antigen processing (Griffin et. al., 1993), decrease in proliferation of lymphocytes (Correa et al., 1998) and interleukin-2, which may be responsible for or be an indication of limited clonal expansion of autoreactive T cells (Griffin et. al., 1993). It has also been demonstrated that the protective effect of RST is more profound when restraint stress was imposed on animals during the expansion period of autoreactive T cells. Yet no

suppression was noted if RST was administrated after the relapse of EAE, when the T cells were already expanded (Whitacre et al., 1998). The net effect of stress on immune function is largely determined by the timing at which the stress was introduced and the stage at which the immune response has developed against the antigen.

An aggravation, not an attenuation as seen in EAE, in the disease severity was found in the Theiler's virus model when RST was applied during early infection. The induction of EAE (as indicated by paralysis) takes much a shorter time (about 10 days) than demyelination induced in the TVID model, which usually requires months and occurs in the chronic stage of disease. In TVID, the first phase of the immune response targets TMEV, which produces virus-specific T cell against viral peptides (VP) and lasts throughout the course of disease. The second immune response, epitope spreading, occurs possibly due to bystander damage of TMEV-infected oligodendrocytes and results in releasing of endogenous autoneuroantigen and generation of myelin-specific T cells, which are responsible for the propagation of demyelination. The antimyelin T cells are generated after the second month post infection in a sequential order (Tompkins et al., 2002). We previously reported that RST produces a profound immunosuppression and consequently an impaired viral clearance (Campbell et al., 2001; Mi et al., 2004, Welsh et al., 2004). In the present chronic stress paradigm, 4 week RST appeared to temporally suppress the immune response to virus infection and may not impact the antimyelin response which usually initiated 2 months p.i. However, increased viral load by chronic restraint stress may hasten the subsequent epitope spreading and lead to an earlier onset, higher incidence of demyelination and more severe disease in this study.

The results demonstrated that acute RST (2 h) initially delayed the onset, and then

the disease progressed as a similar rate as the non-restrained mice (after week 18 p.i.), but exhibited a similar degree of disease severity with all the other two groups. (Figure 5-1 and 5-2). The higher incidence of demyelination (Table 5-1) further suggested that the 2 h RST exerted an exacerbating effect on the disease, but at a lesser degree than that of the chronic RST. The data were at variance with others which reported an immunoenhancing effect of acute stress (Bilbo et al., 2002; Dhabhar and McEwen, 1997), which would be expected to result in a more efficient viral clearance and consequently an attenuated demyelination. Acute stress has been reported to result in leukocyte redistribution from the circulation to the skin, lymph nodes, lungs, liver and bone marrow, so as to establish local immunity, for example, an enhanced DTH response (Dhabhar, 2002, 2003; Dhabhar and McEwen, 1997). The increase in cell trafficking to those organs may lead to a reduction of leukocytes entering the CNS. It may also be argued that acute stress increases the permeability of blood-brain-barrier (BBB) by mast cell degranulation (Esposito et al., 2001), resulting in an influx of leukocytes into the brain. However, due to the distribution of mast cells in the brain, those areas, such as the diencephalons, cerebellum and brain stem, which have a greater number of mast cells, showed an increased permeability following stress; whereas the cortex, the site for virus injection, only showed a minimal change (Esposito et al., 2001). We speculate that the 2 h session RST may have an overall suppressive effect similarly as with chronic RST, but to a lesser degree. This interpretation of our results is indeed supported by other studies showing that either acute 15-min RST (Goujon et al., 1995), 15 min swim stress (Connor et al., 2005) or foot shock (Meltzer et al., 2004) inhibit lipopolysaccharide (LPS)-induced proinflammatory cytokines production. These results taken together indicate that the

intensity, duration, and the experimental animals are key factors in determining the effects of stress.

The stress hormones are believed to contribute to the bidirectional effect of stress. In this study, we measured the CORT level immediately after the cessation of 2 h restraint stress of acute restraint and the first 8 h of chronic restraint sessions. 8 h restraint stress results in a greater elevation (13 fold) than 2 h RST (2.5 fold) over baseline CORT level. We have shown that the CORT level remained elevated until the cessations of RST, indicating that the chronically restrained mice were repeatedly exposed to high CORT levels, even though there was a decrease in CORT during last two weeks of the 4-week periods of RST (Sieve et al., 2004). The immunosuppression as a result of long-term elevated CORT exposure can be explained by the inhibitive functions of glucocorticoid, including antigen presentation, T and B cell activation, proliferation, cell trafficking and proinflammatory cytokine production (Sapolsky et al., 2000). Brief and low-doses of CORT treatment, rather than long or high-doses of CORT can enhance skin DTH response (Dhabhar and McEwen, 1999), suggesting the duration and dosage of CORT determines its effect. It has been reported that plasma CORT levels peaked at 30 minutes following RST and deceased but remained at levels greater than the baseline when the 2 h RST ended. Subsequently, within 3 hours after the cessation of restraint stress, CORT levels returned to baseline or below baseline, and were accompanied by a reversal of the decrease in number of blood leukocytes (Dhabhar et al., 1994). In our model, was the CORT level during acute RST in the range that caused immunoenhancement? Or was the duration of exposure to the elevated CORT levels (before it dropped below baseline) appropriate for promoting the immune function? The questions remain to be answered.

Other groups have reported that plasma catecholamines (epinephrine and norepinephrine) were increased by the end of 2 h RST, and were reduced to baseline within 2 hours (Kanemi et al., 2005). This elevation was also observed in the first 2 weeks of chronic stress (Silberman et al., 2003), which lasted for a shorter time frame than the CORT response, indicating that the SNS axis contributes more to the immune regulation in the acute stress than in chronic stress response. This concept is supported by the finding that an increased susceptibility to *Listeria monocytogenes* was observed following a combination of acute cold and restraint stress, which was mediated by β1-adrenaergic receptors (Cao et al., 2003). Meltzer et al. (2004) found that stress-induced immunosuppression seemed to be mediated by both HPA and SNS axes. Moreover, other stress hormones, ACTH (Conti et al., 2000), CRH (Elenkov et al., 1999) and prolactin (Matalka, 2003) are additionally implicated in this complicated stress response.

The histopathology confirmed the finding that the maximal aggravating effect of chronic restraint stress on the demyelination, such that axonal loss was observed, and a notable demyelination with prominent inflammation in acutely stressed mice, and minimal lesions were noted in the non-restrained mice. The scarcity of inflammatory cells in chronic restrained mice suggest a later stage of demyelination at day 220 p.i., at which time the inflammatory cells may have damaged the myelin and axon and exit the spinal cord. In fact, our laboratory recorded an increased cellularity and inflammation in the chronically restrained mice at day 127 p.i. as compared to non-restrained mice (Sieve et al., 2004). Taken together with the current study, we observed a dynamic progression of demyelination. However, a lower incidence of demyelination was noted in this study, which is possibly due to the fact that the mice used were bred in our animal facility and

had not been subjected to transport stress.

The suppressive effects of both the acute and chronic stress on the humoral response were demonstrated by a strikingly decreased anti-TMEV antibody production throughout the course of disease. The serum antibody was detected as early at day 7 p.i. (data not shown), and remained at a relatively high level throughout the disease. Whilst the restrained animals only displayed baseline levels of TMEV antibody. A significantly higher anti-TMEV IgG was noted at day 28 and day 220 p.i., indicating stress impacted the humoral response in both the phases of acute and chronic disease. We observed a minimal level of serum antibody against MBP at day 220 p.i., with a slightly but not statisticall higher level, in non-restrained/infected than either acute or chronic restrained/infected mice. At this time point, no $PLP_{139-151}$ or MOG_{33-35} antibody was detected in the serum of any mice. The detection of serum autoantibody to MBP and the myelin membrane during the chronic phase was previously reported (Rauch et al., 1987; Sieve et al., 2004; Welsh et al., 1987). However, the humoral immune response plays a major role in viral clearance during the acute disease (Rossi et al., 1991), and only a minor role during the chronic demyelination, but an ameliorating effect was suggested (Rodriguez et al., 1988).

Very interestingly, two chronic restrained, uninfected and one acute restrained, uninfected mice developed increased anti-MBP antibody in the serum at day 220 p.i.. A recent study described a possible scenario for the pathogenesis of autoimmune disease in the NOD model of diabetes. It has been shown that mice present with lymphopenia and the remaining T cells undergo vigorous compensatory proliferation of autoreactive T cells to islet cells (King et al., 2004). Our previous study recorded lymphopenia in stressed

mice (Campbell et al., 2001); therefore we speculate that restraint stress-induced lymphopenia provides the environment necessary for the homeostatic expansion of autoreactive T cells. Autoantibodies to myelin have also been detected in normal human subjects (Johnson et al., 1986). However, the fact that these animals in our study did not develop demyelinating disease may suggest a more complex explanation for this phenomena which would include the underlying increase in autoreactivity that occurs as a result of the homeostatic expansion of autoreactive T cells, but the fact that they have pre-existing autoantibodies may render them more susceptible to autoimmune disease.

Like the gender effect in MS, there is a gender preference in female mice for developing Theiler's virus induced demyelination but a rapid deterioration in disease severity is noted in males (Alley et al., 2003; Hill et al., 1998). We found a higher incidence of demyelination in males than females in this study. However, the overall incidence of demyelination is low in this study, the gender effect on demyelination may be biased by the small number of animals. The gender variance can also be attributed to genetic predisposition (Butterfield et al., 2003). Estrogen has been shown to have a protective effect in EAE (reviewed by Offner, 2004), but the role of estrogens in TVID and stress require further investigation.

In conclusion, acute or chronic restraint stress of mice during early infection, resulted in an exacerbation of demyelination and clinical signs. Glucocorticoid, at least in part, may be responsible for the alteration in disease process through suppression of immune function. This study offers experimental evidence demonstrating how stress may influence the pathogenesis of MS. However, due to the nature of TVID, which consists of an acute infectious disease and a chronic autoimmune disease, as well as the

dual role of stress – enhancing susceptibility to infection and ameliorating autoimmune disease, makes it a complicated model for MS and stress. Further study will be focused on the underlying mechanisms for acute stress-induced immune responses.

VI DISCUSSION AND CONCLUSIONS

The series of studies reported herein were aimed at revealing the effect of stress on the pathogenesis of MS using Theiler's virus induced-demyelination (TVID) as a model for MS and restraint stress as an experimental stress model. The main conclusion from this research is that RST applied prior to infection profoundly impacts the progression of both the acute encephalitis and the chronic demyelination.

Restraint stress was shown to suppress the immune function as evidenced by reduced expression of chemokine and proinflammatory cytokines in the brain and spleen, decreased antibody production, and morphological alterations of immune organs - atrophy of spleen and thymus. The reduction in chemokine expression may account for the decreased inflammatory cell infiltrates in the brain. The decreased pro-inflammatory cytokines may contribute, in part, to increased levels of virus within the CNS. The impaired viral clearance alters the course of chronic demyelinating disease as demonstrated by an early onset, increased incidence and exacerbation of demyelination. The increased circulating corticosterone plays a pivotal role in the restraint stress-induced immunosuppression. Additionally, restraint stress facilitates the systemic dissemination of TMEV resulting in increased viral replication in the heart and the development of a cardiotropic variant of TMEV that induces pathology in the heart.

1. Cytokine and chemokine expression in Theiler's virus infection

Chemokines are chemoattractants that serve as signals in recruiting inflammatory cells into inflamed sites. It was shown recently that the binding of a chemokine to the

receptors on the leukocytes attaching to the endothelial cell causes a rapid rearrangement of cytoskeleton and change of cell shape, thus facilitating the migration through the tight junction of vascular endothelium to the infectious sites (Hordijk et al., 2003; Walzer et al., 2005). Previous studies from other laboratories showed a similar pattern of chemokine expression using different strains of Theiler's virus (DA, BeAn, GDVII, H101) and mice (resistant or susceptible). RANTES, IP-10 and MCP-1 were consistently detected in the CNS (brain and spinal cord) of DA-infected B10.M (susceptible, H-2^f) and B10 (resistant, H-2^b) mice, as well as in BeAn-infected SJL/J mice during early infection (Hoffman et al., 1999; Murray et al., 2000), Another group also recorded that the same chemokine panel containing a greater number of cytokines, RANTES, MCP-1, IP-10, MIP-1β, MIP-1α and β, was observed in SJL/J mice infected with DA, or a more virulent GDVII virus and a less-virulent virus H101 (Theil et. al., 2000). We first reported the chemokine expression of CBA mice that are of intermediate susceptibility to TVID and showed an increase of Ltn, RANTES, IP-10, MCP-1 and TCA-3 in the brain. The differences reported by various laboratories may be due to different techniques used for measuring chemokines. The first two groups used RT-PCR, and Theil's and ours used RPA, which included a greater number of chemokines in the commercial kit. The finding that a common panel of IP-10, RANTES and MCP-1 was found in all the stains of mice infected with various strains of virus suggests that the initial chemokine response to Theiler's virus infection is independent of virulence of the virus and the genetic susceptibility of mice during acute infection. However, the chemokines remained elevated in the spinal cord of susceptible B10.M, but returned to the baseline in resistant B10 at day 21 p.i., the time when virus is cleared from the resistant mice (Murray et al., 2000), indicating the chemokine

expression is correlated with virus levels or the degree of inflammatory infiltrates; whereas a resurgence was observed in the spinal cord of B10.M during the chronic phase (Murray et al., 2000) thus reflecting the degree of chemokine-governed infiltrates which is responsible for demyelination or persistent virus infection in susceptible mice. The chemokine measurements enable us to look into the extent of inflammatory migration both temporally and spatially in this Theiler's virus model. Indeed, we also noted chemokine expressions in the spleen, suggesting an early viremia occurs and the systemic spread of virus in the lymphoid organs. The existence of virus in the spleen was confirmed by plaque assays (see chapter IV).

Cytokines, effector molecules produced by immune cells, play an important role in numerous aspects of the immune response involving stimulation, activation, proliferation of immune cells, and direct antiviral activity. Cytokine changes following Theiler's virus infection have been documented using various strains of virus and mice. It has been shown that both susceptible and resistant mice displayed an increased expression of the panel of pro-inflammatory cytokines, including TNF-α, IFN-γ, IL-1β, IL-12, IL-6 in the brain during the acute phase, which is in accordance with our findings in the CBA mice. The difference in cytokine expression was seen in the chronic phase at which higher pro-inflammatory Th1 cytokines, especially TNF-α, IFN-γ and IL-6 were observed in the spinal cord of susceptible SJL/J mice (DA or BeAn infected) than resistant B6 mice (Chang et al., 2000; Sato et al., 1997; Troitter et al., 2004). A study comparing the cytokine levels in BeAn-infected SJL/J and PLP immunized EAE suggested that TNF-α and IFN-γ were correlated with infiltration CD4+ T cell and F4/80+ macrophages as well as clinical signs. (Begolka et al., 1998). Greater viral load was shown to be associated

with elevated cytokine expression in the CNS (Sato et al., 1997; Troitter et al., 2004). The virulence of virus seems not to be related to the pattern of cytokine in the CNS (Theil et al., 2000). It is suggested that the level of cytokines, but not the type, is responsible for the major difference in viral clearance between susceptible or resistant strains of mice. Anti-inflammatory Th2 cytokines were expressed in susceptible mice (Sato et al., 1997; Begolka et al., 1997), but did not correlate with susceptibility or resistance. We found no virus induced-Th2 cytokines in CBA mice; this could be due to different strain of mice used in our study. The variance of cytokine expression was also noticed in certain mice (Troitter et al., 2004). In addition, cytokine expression was shown to parallel with chemokine expression (Troitter et al, 2004), thereby, cytokine assessment may reflect the degree of activation of the immune response, and the chemokine level as well. The determination of cytokine or chemokine level can be indicative of both innate and adaptive immune response to Theiler's virus infection, and suggestive of viral clearance in the CNS, which further determines the progression of demyelination, due to the close association of their expression with viral load in this model.

2. Restraint stress suppresses the immune response and increases the susceptibility to infectious diseases

The present study demonstrated a profound suppression by restraint stress on the immune response to the early Theiler's infection. The reduction in chemokine expression of Ltn, IP-10 and RANTES, which were demonstrated as chemoattractant for activated T cells, monocytes and NK cells (Giancarlo et al., 1996; Hedrick et al., 1997; Kelnel et al., 1994; Salmaggi et al., 2002) may be responsible for the decreased inflammation in the

CNS following restraint stress. The decrease in pro-inflammatory Th1 cytokines by restraint stress may be interpreted as diminished cellular sources of cytokines resulting from decreased cell trafficking in the CNS or glucocorticoid-induced reduction in lymphadenopathy of spleen, thymus and draining lymphoid nodes (Tarcic et al., 1998; Hermann et al., 1995). Expression of IL-12 p40, a key cytokine driving Th1 response in Theiler's virus infection, declined in the restrained mice, which may contribute further to reduced Th1 cytokine production. The decreased Th1 cytokines, TNF-α, IFN-γ and IL-6 were shown to be correlated with increased viral load within the brain. Together with our previous results, which showed a decreased NK cell activity by restraint stress (Welsh et. al., 2004), the current study reinforces the strikingly suppressive effect of stress on the exacerbation of early Theiler's virus infection, as evidenced by increased mortality (Campbell et al., 2001), and increased CNS viral replication (Campbell et. al., 2001 and this study).

The stress-induced negative effect on immune function was also verified using other virus models. Sheridan and colleagues employing influenza virus showed that RST impaired both chemokine expressions of MCP-1 and MIP-1α (Hunzeker et al., 2004) and cytokine production of IL-1, TNF-α and IL-6 in the lung (Konstantinos and Sheridan, 2001; Sheridan et al., 1991; Sheridan et al., 2000). A delayed infiltration in the lung and decreased NK cell activity were also noted in the lung (Hunzeker et al., 2004; Tseng et al., 2004). Consistent with their findings (Hunzeker et al., 2004), we observed an increase of type I interferon β induced by RST, which may be explained by a compensatory response and/or adaptive response to restraint stress. The level of IL-1β was not significantly changed by RST in the brain in Theiler's virus infection, which is in contrast to influenza

infections in the lung. Unlike the lung, in which inflammatory cells primarily contribute to IL-1 production, the hypothalamus is the main source for CNS IL-1, the production of which was not impacted by restraint stress (Deak et al., 2004). Since cytokines are produced by the CNS, immune system and endocrine organs, as well as the extensive distribution of cytokine receptors between these systems, cytokines are considered as a common mediator for communication within the systems. The stress response involves all the three systems so that cytokines induced by stress may interact with the infection-cytokines within the CNS, thus making the cytokine response in the CNS more intricate than other systems.

Previous research has shown that TMEV infection upregulates toll-like receptor (TLR) expression on microglia and subsequent production of proinflammatory cytokine and chemokine expression, and that stimulation of TLR also increased MHC class II and costimulatory molecules, thus inducing APC function of CNS microglia (Olson and Miller, 2004). Accordingly, during restraint stress, increased viral load may have promoted APC function and viral clearance. However, the predominately immunosuppression of stress was evidenced by the findings in this study. The contradiction can be explained by the increased release of GC induced by stress, which have been found to bi-directionally regulate TLR expression, such that TLR-4 and TLR-2 were up-regulated and TLR-3, down-regulated by GC (Galon et al., 2002). TLR-3 has shown to recognize viral dsRNA and the engagement of TLR-3 resulted in activation of NF-κB pathway (Alexopoulou et al., 2001), suggesting a decreased stimulation of TLR during stress and thus inhibiting antigen presentation. GC-mediated regulation in antigen presentation has been extensively studied in dentritic cells (DC). For example,

dexamethosone inhibit maturation of DC by down-regulating costimulatory molecules and antigen presenting molecules (Matasic et al., 1999; Ruemmele et al., 1999; Pan et al., 2001), and clonal expansion of DC (Matsue et al., 2002). Yet the endocytoxicity of DC was not affected by dexmethesone (Pan et al., 2001). It was recently reported that CORT inhibited the formation of viral peptide-MHC I class complex in DC, and further decreased activation of T cells (Tryckenmiller et al., 2005). Literature concerning the effects ofGC on microgila is sparse; however, the findings from DC implicated that TMEV infection-induced activation of microglia may be partially counteracted by CORT during restraint stress, and may further contribute to the decreased proinflammatory cytokines through inhibition of TLR mediated NF-κB pathway. In fact, GC was also reported to directly inhibit iNOS, NO and TNF-α production of microglia (Chang and Liu, 2000; Drew and Chavis, 2000).

The down-regulation of immune response in lymphoid organ by restraint stress was additionally substantiated in herpes simplex virus (HSV-6) infection. Cellular immunity, NK cell and HSV-specific CTL, virus-specific memory CTL (CTLm) were suppressed by restraint stress (Bonneau et al., 1991a; Bonneau et al., 1993). Restraint-induced decrease in IL-2 production results in a slowdown in the proliferation of CTLm in the lymph nodes (Bonneau et al., 1991b). The reduction in cytokine production was noted in the spleen, which accounted for the decreased splenic HSV-specific CTLm (Bonneau, 1996). It is noted that this HSV model demonstrated stress-induced immunoregulation using a neurotropic virus infection, which is in accordance with our report, that restraint stress exacerbated HSV-induced encephalitis and increased mortality, with decreased CNS cellularity (DeLano and Mallery, 1998) and a delayed HSV-specific $CD8^+$ response in the

CNS (Anglen et al., 2003).

Further investigation of infection in extraneural organs in our study revealed an enhanced viral replication in the heart, spleen and cervical lymph nodes after stress, suggesting a facilitated systemic infection of Theiler's virus possibly by elevated corticosterone following restraint stress. Since the viral antigen can be detected in systemic organs up to 4 month in non-stressed mice (Trottier et al., 2002), we speculated that the increased viral replication in the stressed mice may prolong the persistence of virus in these organs and therefore these sites may become additional sources of virus for repeated CNS infection.

The present study substantiated the fact that restraint stress compromises antiviral immunity, resulting in impaired viral clearance in the CNS, and also contributed additional experimental evidence to the notion that stress increases the susceptibility to infectious disease, including any candidate virus for MS. Additionally, the altered tropism of Theiler's virus to the heart following stress, implies a novel infectious disease could develop as result of stress-induced immunosuppression in the organs that are not normally affected.

3. Restraint stress exacerbates chronic demyelination in Theiler's virus infection

To our knowledge, we are the first laboratory ever to study the stress and demyelination in a virus model. The present study provides laboratory data demonstrating that stress can aggravate demyelination which is in agreement with clinical observations and epidemiological evidence that stressful life events are associated with exacerbation of MS (Ackerman et al., 2002; Mohr et al., 2004; Strenge et al., 2001).

Therefore we conclude that the impaired antiviral immunity caused by RST allow the establishment of viral persistence and increase viral load in the CNS, which produces exacerbation of demyelination. The change can be attributed, at least in part, to the sustained elevation of corticosterone over the stress period (Campbell et al., 2001; Sieve et al., 2004). The magnitude of increase in corticosterone can decline with exposure time, which is due to blunted HPA axis activity resulting from habituation or adaptation of animals to repeated chronic stress (Bauer, et al., 2001; Pecoraro et al., 2004). The stress effect can remain as long as 10 days after the cessation of stress (Whitacre et al., 1998). Therefore, the chronic stress may profoundly impair the immune function for a considerable length of time.

The observations of the association between MS and stress do not always agree. For example, a study reported an attenuation of MS after stress exposure (Nisipeanu and Korczyn; 1993). Some MS patients have psychological symptoms, which might be the result but not the cause of exacerbation (Mohr and Cox, 2001). The inconsistency between studies suggests that complex and differential mechanisms of stress, as well as various categories of MS patients, relapsing-remitting MS (RRMS) or primary progressive MS (PPMS), may determine the outcome of the course and progression of demyelinating disease. For example, autopsy studies revealed that MS patients have more CRH/AVP neurons and higher CRH mRNA in the hypothalamus than healthy controls (Erkut et al., 1995; Huitinga et al., 2004), indicating activation of these neurons in MS patients. However, the severity of disease was related to the reduction in the number and activity of CRH/AVP neurons (Huitinga et al., 2004). These findings implicate the role of HPA axis (not related to stress) in MS and possibly an altered HPA axis responsiveness

at the stress exposure. It has been found that the different HPA responsiveness in MS patients, with the order from the most to the least hyperreactive noted in primary progressive MS (PPMS), secondary progressive MS (SPMS) and relapsing-remitting MS (RRMS) patients (Morh, personal communication). This may explain the different consequences of stress effect on MS.

Stress and demyelination are indeed two conditions that display different kinetics of development and progression, and are regulated by different but interactive mechanisms. The HPA axis is believed to be the core factor involving both processes. The outcome is dependent on many factors, such as the intensity, duration of stress, and the interval between the onset of stress and demyelination. For example, in the EAE model, stress prior to immunization ameliorated the disease, but not after the challenge (Correa et al., 1998; Whitacre et al., 1998). Acute stress was shown to be immunostimulating (Dhabhar and McEwen 1997), therefore we might expect an enhancing effect of acute restraint stress in the TVID model, due to activation of the immune response, increased viral clearance and subsequent attenuated demyelination. However, the results showed a similar, but less suppressive, effect in the immune function, which resulted in an intermediate exacerbation of demyelination. This requires a detailed dissection of the stress paradigm in terms of intensity, duration and the kinetics of stress hormone, CORT as well as catecholamines. Additionally, CNS infection itself is a stressor that may impact the neuroendocrine circuit, thus making the stress-MS relation more intricate. However, the current study provides a model system with relevance to MS onset of those patients whose disease is triggered by virus infection during a stressful life event. The establishment and effectiveness of this stress-MS model enables us to scrutinize the

endocrine and immune response in the development of demyelination and eventually shed light on the neuropathogenesis of MS.

4. Future studies

The key in the Theiler's model is the establishment of persistent CNS infection and we are interested in the how stress affects the efficacy of the immune system in viral clearance. For future studies, we will focus on the following aspects:

1) We showed decreased inflammation by stress at acute infection and selective reduction of chemokine expression; therefore, we will need to identify the phenotype of cellularity affected by restraint stress. We will further focus on macrophage and microglia, because these cells are the major antigen presenting cells and the reservoir for harboring the virus in the chronic disease. It is very important to examine how stress impacts viral antigen presentation at the early stage as well as myelin antigen presentation by these cells and astrocytes. The identification dynamics of the changes by stress is necessary for further investigation of the effect of on the two distinct processes.

2) We have demonstrated that pro-inflammatory cytokines are associated with viral clearance. The profile of cytokine expression, especially in the spinal cord, needs to be identified. Apart from virus load, the cytokine level is expected to be linked to demyelination, which is mediated by Th1 cytokines. Since stress may exacerbate the demyelination (or possibly attenuated by acute stress, if we could find an appropriate paradigm for acute stress and the hypothesis is true in this model), an enhanced Th1 cytokine (or decreased by acute stress, if any) expression is expected.

3) Optimization of the duration, intensity and the timing introduced into the animal

(interval between infection and stress) for acute stress procedure. It will be necessary, first, to identify the dynamics of release of stress hormone in response to acute stress, second, determine the consequence of stress by examining the viral clearance in the CNS following stress, especially around the initiation of demyelinating disease, and a more sensitive technique may be required.

4) For the chronic stress, we observed a behavioral habituation to stress in the animals, as well as a diminished suppressive effect in the spleen, which may indicate an HPA responsiveness adaptation or blunted HPA axis activity by stress. CRH has been suggested in the chronic stress adaptation; the role of this particular hormone, as well as ACTH in this model remains to be investigated.

5) It is very interesting to notice that stress may even alter the tropism of virus and cause a novel disease. In addition to altered immune response by stress which allows a novel infection, the nature of the virus (changed or not), and the receptors for the entry of virus (changed by stress hormone or utilizing different receptor) also need to be examined. This may give us a clue as to whether stress may alter the pathogenecity of a virus and increase the susceptibility of human or animal to some infectious disease which is usually less pathogenic.

6) Another important indication from the current study is that some uninfected chronic and acute stressed mice developed autoantibodies to myelin. It may suggest an autoimmune mechanism for demyelination in MS. Extensive and detailed research will be required to dissect the T cell response to myelin antigen following stress.

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Mi, W., Young, C.R., Storts, R.W., Steelman, A.J., Meagher, M.W. and Welsh, C.J.R. Restraint stress facilitates systemic dissemination of Theiler's virus and alters its pathogenecity. (submitted)

Meagher, M. W., Belyavskyi, M., Sieve, A. N., Mi, W., Johnson, R.R., Welsh, T. H and Welsh, C.J.R. Maternal separation disrupts viral clearance and exacerbates the acute phase of Theiler's virus infection in adult mice. (submitted)

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