DIFFERENTIAL EFFECTS OF STRESS ON A MURINE MODEL OF MUTIPLE SCLEROSIS

An Honors Fellows Thesis

by

JESSICA JEAN HEIBEL

Submitted to the Honors Programs Office
Texas A&M University
in partial fulfillment of the requirements for the designation as

HONORS UNDERGRADUATE RESEARCH FELLOW

April 2011

Major: Biomedical Science

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Research Advisor: C. Jane R. Welsh
Associate Director of the Honors Programs Office: Dave A. Louis

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ABSTRACT

Differential Effects of Stress on a Murine Model of Multiple Sclerosis. (April 2011)

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Multiple sclerosis (MS) is a demyelinating autoimmune disease that affects the central nervous system (CNS) in humans. Several studies have shown a strong correlation between stressful events and the onset and exacerbation of MS in patients. Based on this information, similar studies have been undertaken in mice. CNS demyelination is induced in mice by infecting them with Theiler's murine encephalomyelitis virus (TMEV). After the initial encephalitis phase, the virus persists in susceptible mice strains and demyelination of the CNS occurs, creating a useful model of MS.
Using this model, several types of stress – social, restraint, and handling – have been utilized, either prior to infection or concurrently, to study the effects in virus-induced demyelination in mice. However, these studies have primarily focused on the effects of chronic stress, while the effects of acute stress on a MS model have, for the most part, been ignored.

The objectives of this experiment are to examine the differences in immune response between chronically and acutely stressed mice. Mice in the acute stress group are hypothesized to experience an enhanced immune response, which should lead to: better viral clearance, smaller and fewer lesions in the CNS, and better physical coordination than the chronic stress group.

Mice will be separated into three groups. One group will undergo chronic stress, another will undergo acute stress, and the last group will serve as the control. Mice will be infected with TMEV and monitored for effects. Weight measurements and behavioral scoring will be utilized as a way of monitoring disease progression in live mice. Continual monitoring will continue as TMEV is allowed to persist into the chronic, demyelinating phase. The mice will be terminated to collect tissue and serum samples during this phase.

Since studies comparing immunological effects of acute versus chronic stress have consistently shown that immunosuppression is associated with chronic stress, while immunoenhancement is associated with acute stress, polar results are also expected in this experiment. Following TMEV infection and subsequent CNS demyelination, different results between the chronically and acutely stressed mice seem likely.

DEDICATION

This thesis is dedicated to my family, especially to the members who will read every single page of this out of love, without understanding half of it; take pride, because I couldn’t have written any of this without you.

ACKNOWLEDGMENTS

Dr. Jane Welsh has my utmost gratitude for mentoring me during the past two years of my undergraduate career. Without her enthusiasm and generosity, I would not have become so involved in research. I would also like to thank Dr. Colin Young who was always available for me to question about research or current events. His advice has proven invaluable over the course of this thesis. In addition, Francisco Gomez provided excellent assistance and guidance when I was reviewing slides. For technical assistance, I would like to thank Krystin Deason and Christina Dudash, whose assistance prevented me from becoming too overwhelmed. Finally, my appreciation is given to Dr. Dave Louis and the Honors Office whose classes helped prepare me to write and prepare my thesis.

Once again, thank you to everyone who has had even the slightest part in this project; it would not have been possible without you.

NOMENCLATURE

ARS	Acute restraint stress
BBB	Blood brain barrier
CNS	Central nervous system
CRS	Chronic restraint stress
EBV	Epstein-Barr virus
HLA	Human leukocyte antigens
HPA	hypothalamo-pituitary-adrenocortical
I&D	Inflammation and demyelination
MBP	Myelin basic protein
MHC	Major histocompatibility complex
MOG	Myelin oligodendrocyte glycoprotein
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
NK cell	Natural killer cell
NRS	No restraint stress
PBS	Phosphate buffered solution
PFU	Plaque forming units
PI	Post infection
PPMS	Primary progressive multiple sclerosis
RRMS	Relapsing remitting multiple sclerosis

RS	Restraint stress
SNS	Sympathetic nervous system
SPMS	Secondary progressive multiple sclerosis
TMEV	Theiler’s murine encephalomyelitis virus
TNFα	Tumor necrosis factor alpha
TVID	Theiler’s virus-induced demyelination
WM	White matter

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CHAPTER I

INTRODUCTION

Multiple sclerosis

MS is the most common autoimmune disease of the central nervous system and is characterized by demyelination of the CNS. In the United States, approximately 250,000 to 350,000 people are affected by this disease (Noseworthy et al., 2000). Diagnosis typically involves patient history of symptoms and magnetic resonance imaging (MRI) to detect lesions, with two possible clinical diagnoses: relapsing-remitting MS (RRMS) which is observed in 85-90% of cases, and primary progressive MS (PPMS). Forty percent of RRMS cases fail to recover from attacks over time and develop a form of MS known as secondary progressive (SPMS) (Hafler, 2004).

Although lesions can be found throughout the CNS, certain areas tend to have a greater susceptibility, such as the optic nerve, brainstem, spinal cord, and periventricular regions. The current model for acute lesion development suggests that activated CD4+ T-cells enter the CNS, creating an autoimmune inflammatory reaction. Antibodies against target antigens, such as myelin basic protein (MBP) and myelin oligodendrocyte glycoprotein (MOG), are able to infiltrate the CNS due to the T-cells disruption of the

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This thesis follows the style of Brain, Behavior, and Immunity.

blood-brain barrier (BBB). Once inside the CNS, these antibodies cause either direct or complement-mediated demyelination. Other factors, such as macrophage release of tumor necrosis factor alpha (TNF-α) and interferon gamma are believed to amplify the immune response (French-Constant, 1994, Noseworthy et al., 2000).

The exact etiology of these events is unclear, but many factors are known - or suspected - to be involved in the initiation of MS, including genetic, environmental, and infectious agents. Epidemiological studies show that those of European or Scandinavian descent have a greater susceptibility to developing MS as do first-degree relatives of a MS patient (Kurtzke, 1991, Ramagopalan and Sadovnick, 2011). As technology has advanced, studies have developed a myopic approach and are searching for the specific genetic cause of this disease. Currently, the major histocompatibility complex (MHC) for human leukocyte antigens (HLA) located on chromosome 6 is believed to be the best connection between genetic predisposition and MS (Ramagopalan and Ebers, 2009) although other genes encoding receptors may also have an effect.

Environmental factors such as UV-radiation exposure (Sloka et al., 2011), sex (Ramagopalan and Sadovnick, 2011), and stress (Ackerman et al., 2002) have long been shown to have an effect on disease onset and progression. Epidemiology has been especially useful in elucidating the relationship between these factors and MS. For example, the inverse correlation between UV exposure and MS prevalence has led to the revelation of vitamin D's importance in delaying or reducing symptoms of MS. Also,

sex (characterized as an environmental factor in Ramagopalan's 2009 paper) has been shown to be an important dynamic since females are twice as likely as males to develop MS, a trend common in most autoimmune diseases. Finally, stress has probably been the first such factor connected with MS. In one of the earliest descriptions of MS, Charcot stated that grief, vexation, and adverse changes in social circumstance seemed to be connected to the onset of disease (Charcot, 1877). Further studies have proven a connection between onset and exacerbation of symptoms both in patients and animal models (Ackerman et al., 2002, Mohr et al., 2004).

Infectious agents as a factor in MS development and exacerbation is a topic of debate, however, evidence suggests that viruses such as Epstein-Barr (EBV) could play a role by activating pathogenic T-cells, causing inflammation and subsequent autoimmunity (Franciotta and Lolli, 2005, Lucchinetti et al., 2000). Besides pathological evidence, epidemiological and migrational data also support the idea of an infectious agent being involved in the onset of MS (Kurtzke and Heltberg, 2001, Sloka et al., 2011).

TMEV as a model of MS

First described by Max Theiler, Theiler's murine encephalomyelitis virus causes flaccid hind limb paralysis in mice (Theiler, 1934). The virus consists of two subgroups, the highly fatal and virulent strains, and the less virulent strains, which includes BeAn. Upon intracerebral injection, the BeAn strain causes the acute phase polioencephalomyelitis infection after one week. One month post-infection, the chronic

phase inflammatory demyelinating disease occurs (Tsunoda and Fujinami, 2010). This demyelination is referred to as Theiler's virus-induced demyelination (TVID), and is only seen in susceptible mice such as SJL and CBA strains (Lipton et al., 1986, Tsunoda et al., 1996). TVID resembles MS in that inflammatory demyelination occurs in the white matter (WM) of the CNS coupled with spastic paralysis. Because of the possible involvement of infectious agents in the etiology and exacerbation of MS, TVID has become a useful viral model for the manipulation and study of MS (Olson and Miller, 2009, Sato et al., 2011, Tsunoda and Fujinami, 2010).

Stress

The role of stress in medicine and science was first introduced by Selye. He described stress as the nonspecific response of the body to any demand and the resulting pathological results. He also identified the hypothalamo-pituitary-adrenocortical (HPA) axis as the primary effector of the stress response, and introduced three phases of stress: the alarm, resistance, and exhaustion stages (Selye, 1974). The terms alarm, resistance, and exhaustion are synonymous with eustress, resilience, and distress respectively, while eustress can also be referred to as acute stress and distress as chronic stress (Dhabhar, 2009, Dhabhar and McEwen, 1997). Acute stress is defined as stress during a period of time lasting only minutes to hours, while stress is considered chronic if it lasts for weeks to years (Dhabhar, 2008, Dhabhar and McEwen, 1997, Sheridan et al., 1998). Currently, there are four groups of stressors defined: 1) physical stressors such as heat, cold, noise, etc. 2) psychological stressors such as anxiety, fear, and frustration 3) social stressors

like dominance in animals or divorce and unemployment in humans and 4) exercise, orthostasis, hypoglycemia, etc. which challenge homeostasis in the body (Pacak and Palkovits, 2001).

According to Dhabar and McEwen, acute stress my result in an immunopreparatory or immunoenhancing response, while chronic stress may result in dysregulation or suppression of the immune system (Dhabhar and McEwen, 1997). As reviewed by Kemeny and Schedlowski, during chronic stress an increased release of glucocorticoids due to HPA axis activity coupled with responses of the sympathetic nervous system (SNS) lead to a decrease in cellular and humoral immunity. However, acute stress activates the immune response with higher numbers of natural killer (NK) cell and granulocyte numbers (Kemeny and Schedlowski, 2007). To further elaborate, during acute stress, redistribution of leukocytes occurs in the skin and sentinel lymph nodes, hormone concentrations are at physiologic stress levels, and the source of glucocorticoids remains endogenous, all of which create an immunoenhancing effect. Conversely, chronic stress induces immunosuppression with redistribution of immune cells to leukocyte-depleted compartments such as the blood, hormone concentrations are at extremely high pharmacologic levels, and glucocorticoids are supplied by synthetic analogues (Dhabhar, 2008). Psychological factors such as coping, control, and learning, along with physiological factors which include genetics, nutrition, sleep, and physiological health can influence a person's response to stress, and if well-developed, help maintain homeostasis (Dhabhar, 2009).

Science and medicine are interested in the opposing effects between chronic and acute stress because of the implications towards infection and disease (Dhabhar, 2009, Kemeny and Schedlowski, 2007, Sheridan et al., 1998). Of interest to this study is the previously mentioned relationship between stress and the onset and progression of MS (Ackerman et al., 2002, Mohr et al., 2004, Warren et al., 1982). Restraint stress (RS) has previously been employed with mice to study the effects of chronic stress on the TMEV model of MS (Campbell et al., 2001, Steelman et al., 2009). These studies have shown an exacerbation of disease symptoms, with increases in number of lesions, macrophage infiltration, and clinical severity of disease. (Campbell et al., 2001, Young et al., 2010) In this study, we utilized the TMEV model of multiple sclerosis, to examine how the effects of chronic versus acute restraint stress prior to infection affect the onset of TVID. Because TVID is a viral model of MS, it was hypothesized that the immunoenhancing effects of acute stress will lead to better viral clearance in the mice, which will produce fewer lesions and a better clinical prognosis than their chronic stress counterparts.

CHAPTER II

METHODS AND MATERIALS

Mice

A total of 18 three- to five-week-old SJL mice bred in house were used in these experiments. These mice were assigned to different types of stress accordingly: 7 chronic restraint stress (CRS), 7 acute restraint stress (ARS), and 4 no restraint stress (NRS). For this study, all mice were infected with TMEV. Groups were kept in separate cages, with females and males also separated, and were fed *ad libitum* a diet of mouse chow containing 9% fat 20.5% protein. Water was also provided *ad libitum*. All animal care protocols were in accordance with NIH guidelines for Care and Use of Laboratory Animals and were approved by the Texas A&M University Laboratory Animal Care and Use Committee.

Infection and restraint stress protocol

After anesthesia with isoflurane, mice were injected with 5.0×10^4 plaque forming units (PFU) of BeAn strain of TMEV in 20 µL of DMEM media into the right mid-parietal cortex at a depth of approximately 1.5 mm (Campbell et al., 2001).

Chronic restraint stress was carried out by placing mice in well-ventilated plastic tubes for 8 hours overnight, 5 consecutive nights per week, for a total of 3 weeks (Campbell et al., 2001). The tubes were perforated with small holes and internal diameters ranged

from 2.0-3.0 cm. Acute restraint stress followed a similar protocol, but mice were only restrained once for a period of 2 hours. Infection occurred immediately after the first restraint session. NRS mice were infected concurrently with ARS mice.

Clinical scoring and health monitoring

ARS and NRS mice were monitored daily for 1 week post-infection (PI) and CRS for 3 weeks PI. After these time periods, mice were clinically assessed on a weekly basis. Weights and temperatures were among the data collected during these periods, with a time lapse of 3 hours between restraint stress and weighing for CRS mice.

The other set of data collected was clinical scores, which assesses the following indicators of health in mice: grooming, ruffling, gait, limb strength, hunched back, righting reflex, and general appearance. After considering all of these areas, a single score is given for the mouse's general health. Scores range from 0 to 5, with 0 being a healthy, well-groomed, coordinated and active mouse, while a score of 5 indicates severe disease with complete lack of grooming and response to stimuli, no mobile ability, and spastic, uncoordinated movements.

Termination and histological analysis

Mice were sacrificed 150-162 days PI using a lethal dose of Beuthanasia special 150 mg/kg (Steelman et al., 2009), and were perfused first with phosphate-buffered solution

(PBS) followed by a 10% solution of formalin in PBS. Mice were examined for any gross abnormalities at termination.

After termination, the brains and spinal cords of each mouse were removed, processed, and embedded in paraffin. Tissues were sectioned and mounted on individual slides, then were stained with hematoxylin and eosin (H&E) for microscopic examination.

Spinal sections were read blinded to condition and scored for signs of inflammation (perivascular cuffing, meningitis) and percent demyelination (Sieve et al., 2004). Scores ranged from 0-4 as follows: 0 – lack of inflammation and demyelination (I&D), healthy appearance; 1 – I&D present but limited to 25% or less of WM; 2 – I&D present and limited to 25-50% of WM; 3 – I&D present and extend from 50-75% of WM; 4 – signs of I&D present in 75% or more of section.

CHAPTER III

RESULTS

The effects of restraint stress on clinical scores

As depicted in Figures 1 and 2, CRS exacerbated symptoms related to the acute phase of TVID. Temperatures recorded during the first week PI were lower for all three groups, with NRS and ARS mice exhibiting the greatest decrease in temperature. After the first week, as Figure 1 shows, temperatures returned to baseline and remained through the entire experiment (complete data not shown). Figure 2 shows the general health scores for each group of mice. Scores for CRS mice were elevated compared to ARS and NRS mice, which appeared asymptomatic.

During the 3 week RS period, CRS mice exhibited poor grooming, hunched postures, and severe signs of encephalitis. One mouse was lost during the week 1of CRS, creating n=6 for this group.

RS also caused weight loss in mice during acute phase TVID. CRS appears to have the greatest effect on mice, with an average weight loss of almost 30% according to Figure 3. ARS and NRS also experienced slight weight loss (~15%) but returned to baseline in less than 3 weeks PI (CRS mice returned to baseline ~ 1 month PI).

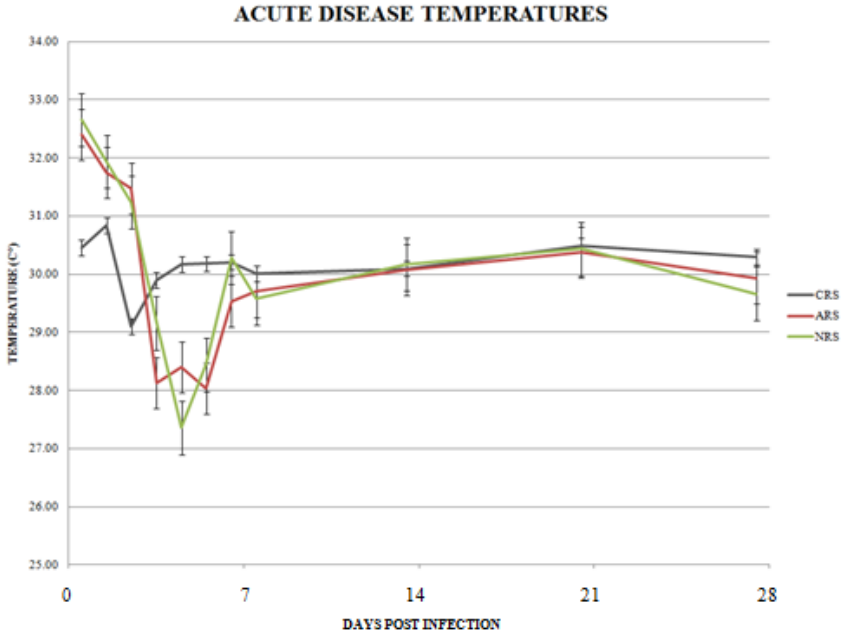

FIG. 1 Acute disease temperatures. Temperatures were recorded of mice for the first month PI. The decline in temperature within days 0-7 PI is an indication of TMEV acute phase disease. Data are expressed as the mean±SEM.

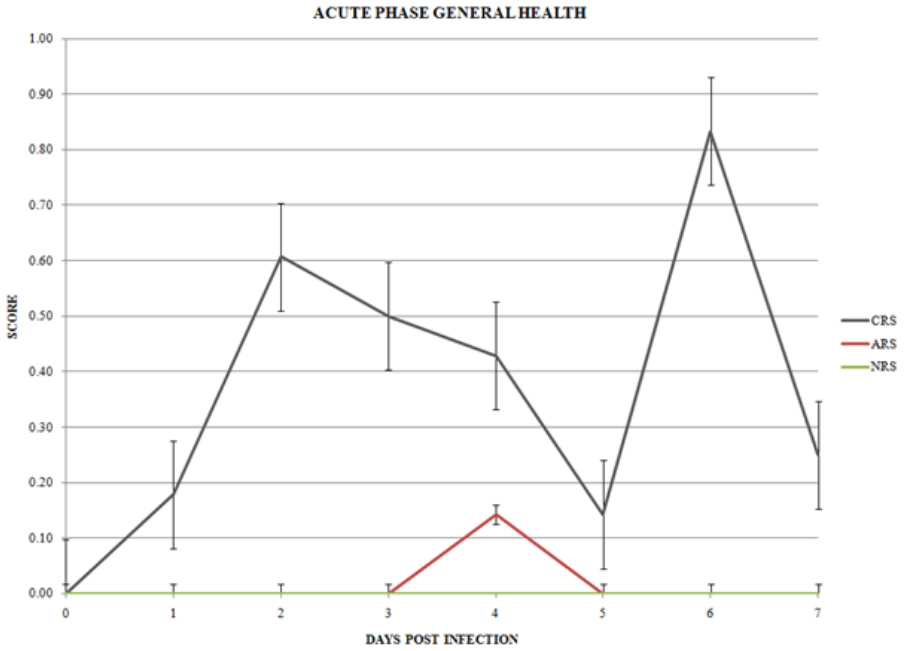

FIG 2. Acute phase general health scores. Clinical scores following TMEV infection during the acute phase of TVID. CRS exacerbated scores while ARS and NRS mice did not display clinical signs in the first week PI. Data are expressed as the mean±SEM.

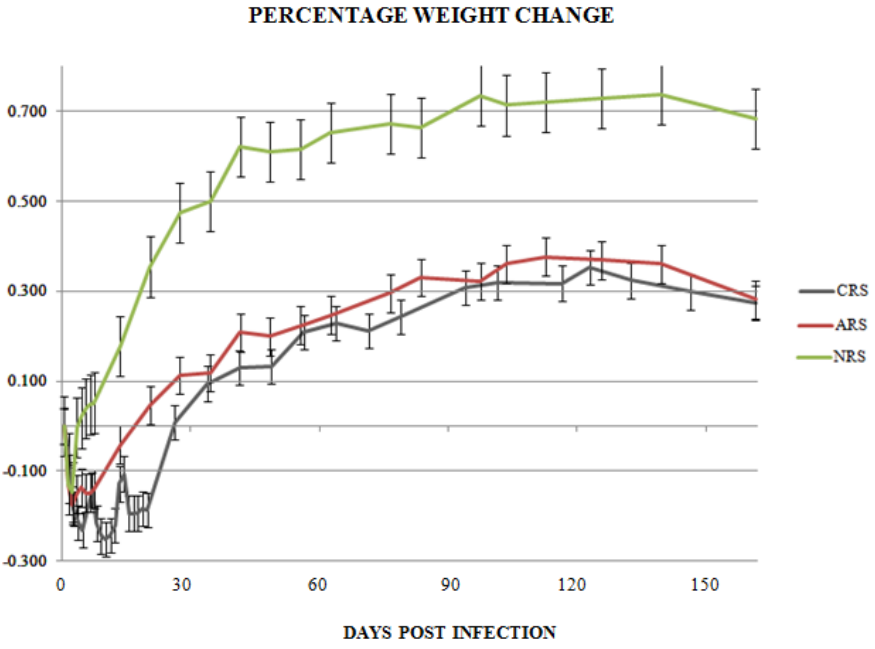

FIG. 3 Percentage weight change. Each group shows an initial weight loss PI, then subsequent weight gain. CRS mice experienced a longer period of weight loss that corresponds to the 3 weeks or RS. Data are expressed as the mean±SEM.

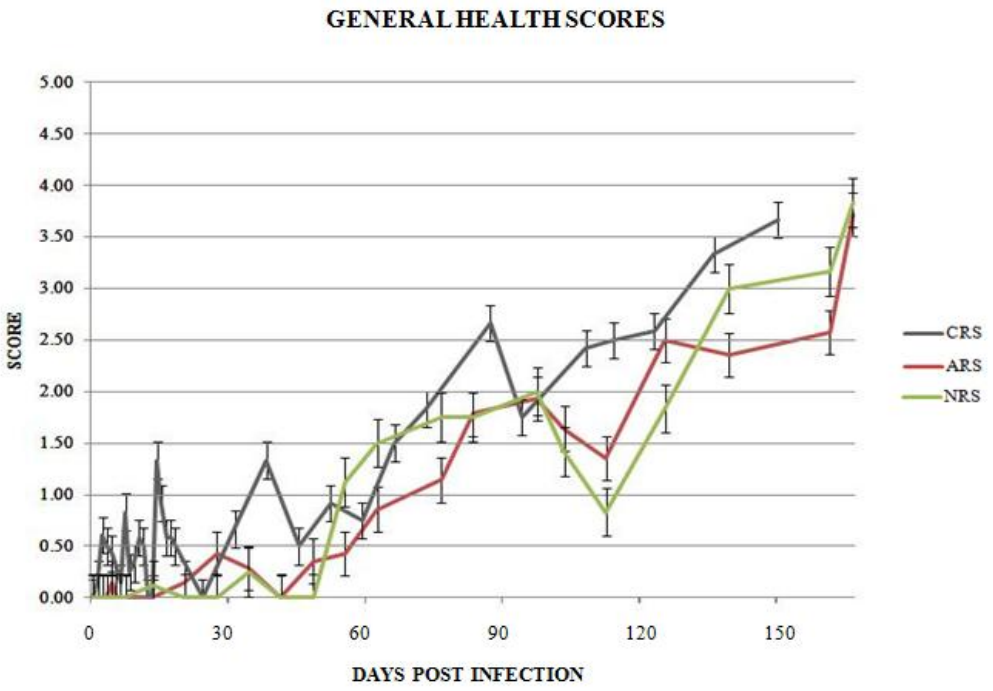

FIG. 4 General health scores Scores are based on a scale of 0-5 with 0 being asymptomatic and 5 being most severe signs of disease. Data are expressed as the mean±SEM

During the chronic phase of TVID, as shown in Figure 3, all mice experienced weight gain. NRS experienced the greatest percentage increase in weight, for which there is 3 possible explanations: 1) RS stunted the growth of ARS and CRS mice 2) NRS mice were the youngest group at time of infection or 3) the NRS group had the highest ratio of males.

Figure 4 displays the general health scores for the entirety of the experiment. As expected, CRS mice had the worst symptoms in comparison to ARS and NRS mice. Although scores between the ARS and NRS groups are similar, NRS mice appeared to develop symptoms of TVID more rapidly than the ARS group between days 45 and 90.

Histological analysis

Figure 5 is a shows the relationship between histology and general health scores. As expected, the CRS group shows a trend to the upper right corner of the plot, indicating severe disease. The histology samples in Figure 6 include one spinal cord section from each RS group. Figure 5-A (CRS) has signs of inflammation, meningitis, and demyelination in ~75% of WM, while Figure 5-C (ARS) shows similar signs in ~40% of its WM. Figure 5-E (NRS) has the least amount of inflammation and demyelination in this sample.

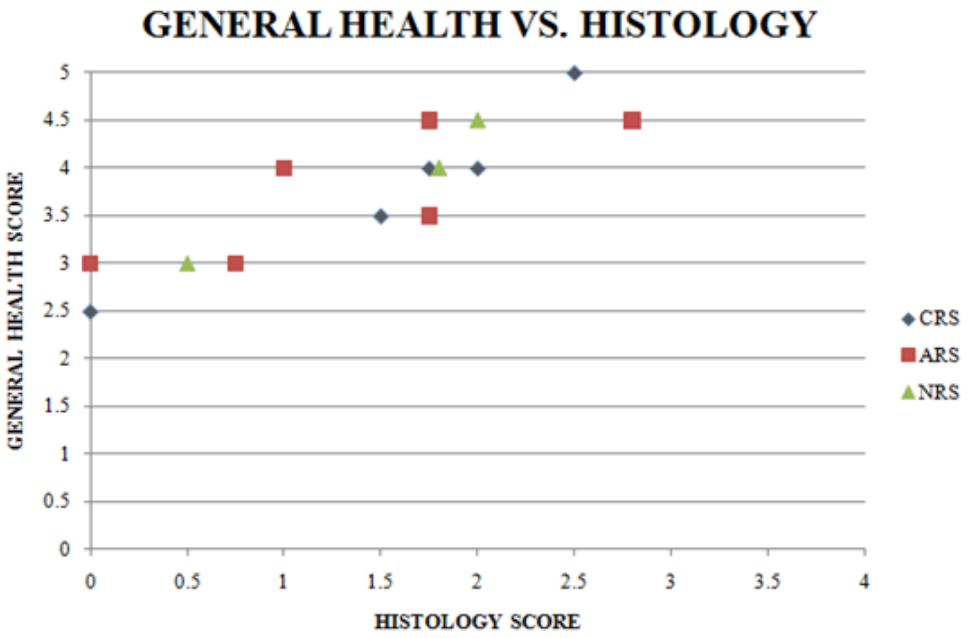

FIG 5. General health vs. histology. Final general health scores were charted against histology scores for individual mice.

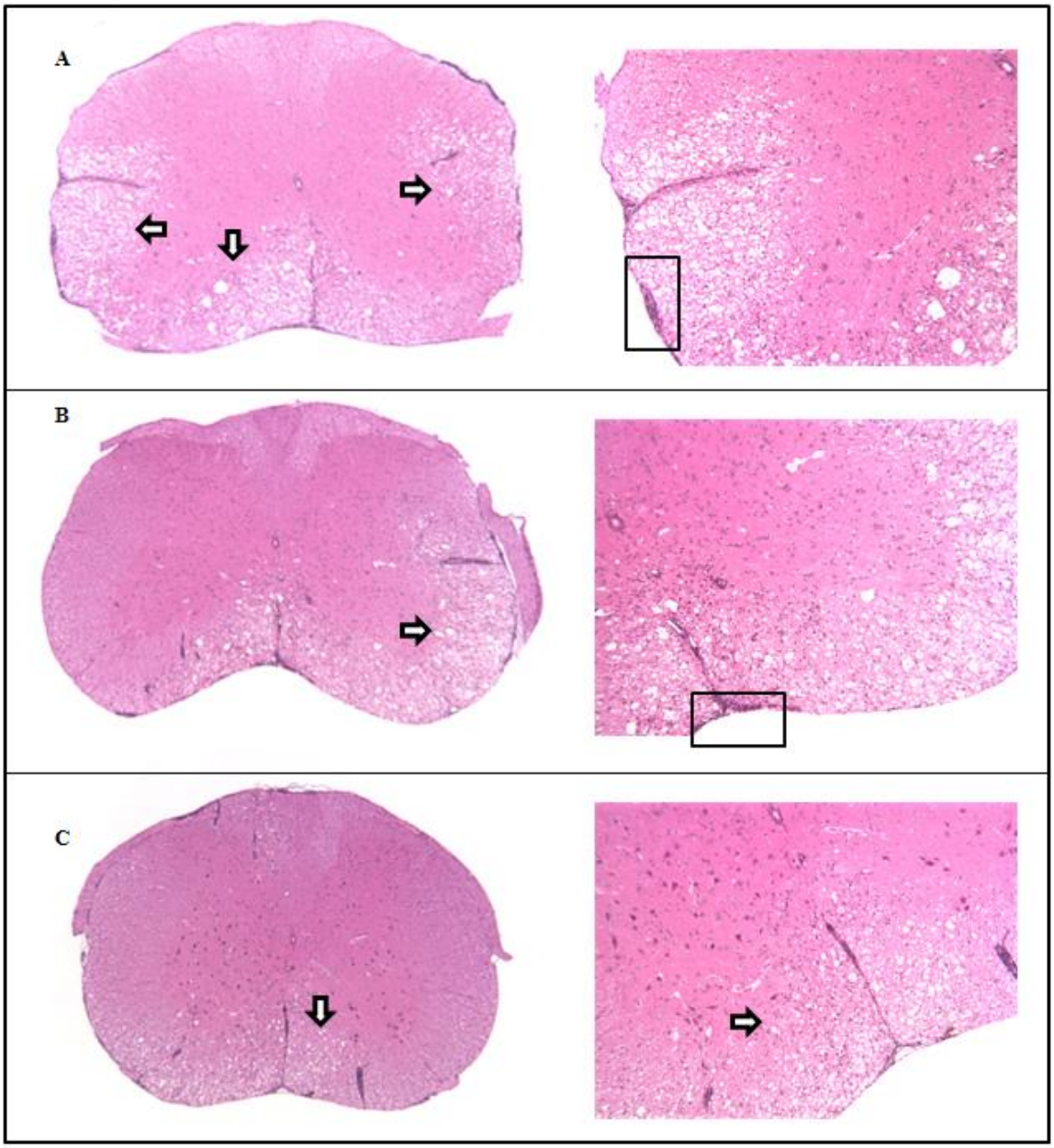

FIG 6. Histology. Representative spinal cord sections stained with H&E. Sections are shown at 4X magnification (left) and 10X magnification (right).

A) Chronic stressed mouse showing extensive demyelination (arrows) and inflammation (boxes).
B) Acute stressed mouse showing less extensive demyelination but increased inflammation.
C) Non-stressed mouse with less demyelination and inflammation than chronically stressed mouse.

CHAPTER IV

DISCUSSION AND SUMMARY

Multiple studies have been conducted to investigate the role of chronic stress on the neuropathogenesis of Theiler's virus in mice. With each study, chronic stress has led to a more severe case of TVID, including decreased body weight and increased clinical scores during the acute phase, and higher clinical and histological scores during chronic disease (Campbell et al., 2001, Sieve et al., 2004, Steelman et al., 2009, Young et al., 2010). This reaction is thought to be mediated by a stress-induced immunosuppression, which, at the onset of TMEV infection, impairs the immune system's ability to clear the virus (Campbell et al., 2001). The virus then replicates to higher levels in the CNS resulting in increased demyelinating disease.

Acute stress enhances the immune system (Dhabhar, 2009, Tsunoda et al., 1996), and we predicted that acute stress prior to Theiler's virus infection would have a protective effect on the disease progression, due to increased viral clearance. Data collected during acute phase TVID indicated that acutely stressed mice were less affected by Theiler's virus than the chronically stressed mice. Chronically stressed mice also experienced a greater percentage of weight loss and higher general health scores, while non-stressed and acutely stressed mice data were similar.

For chronic phase TVID, general health scores were similar to those noted in the acute phase in that the chronically stressed mice were more severely affected. The acutely- and non-stressed mice had similar responses. One important difference between the non-stressed and acutely stressed groups: the clinical scores of the non-stressed mice worsened at a faster rate than the acutely stressed mice's between days 45 and 90. This period is when chronic phase symptoms first became apparent, so this leads to the hypothesis that ARS mice did mount an enhanced immune response, which allowed improved viral clearance and slower disease progression.

When examining histological scores versus final scores for general health, similar trends are once again evident. Considering data of each group, plotted towards the top right corner (indicating most severe disease) is the results of CRS mice. Non-stressed and acutely stressed mice once again show less severe trends, and when compared with each other, the acutely stressed group (excluding outliers) developed the least severe disease. However, preliminary examination of the histology indicated more inflammation and less demyelination in acutely stressed mice.

A weakness in this study was small sample size and lack of statistical analysis and so conclusions drawn are less conservative than desired. Serum was also collected throughout the experiment and stored. Future analysis for GC levels or antibodies could further elucidate differences between the three RS groups. However, based on these results, acute and chronic stress do produce divergent effects in the TMEV model of MS.

Chronic stress is known to cause immunosuppression which impairs the ability to mount an effective immune response to viruses, leading to more severe disease. Acute stress should enhance the ability to clear virus by its immunoenhancing effects. In this study, the clinical observations support this hypothesis in that the acutely stressed mice had a delayed onset of demyelinating disease symptoms. Relating these findings to multiple sclerosis, chronic stress at the time of infection with a pathogen would cause immunosuppression and allow the virus to replicate to higher levels and cause worse late disease. In contrast, acute stress may prove protective in the development of disease. The implication of this study could provide further understanding into how stress may play a role in the onset and establishment of autoimmune disease.

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