NOREPINEPHRINE MEDIATES THE EFFECTS OF

RESTRAINT STRESS ON

THEILER'S MURINE ENCEPHALOMYELITIS VIRUS

A Senior Honors Thesis

By

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Submitted to the Office of Honors Programs
& Academic Scholarships
Texas A&M University
in partial fulfillment of the requirements of the

UNIVERSITY UNDERGRADUATE
RESEARCH FELLOWS

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April 2002

Group: Life Sciences 2

ABSTRACT

NOREPINEPHRINE MEDIATES THE EFFECTS

OF RESTRAINT STRESS ON

THEILER'S MURINE ENCEPHALOMYELITIS VIRUS. (April 2002)

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Theiler's Murine Encephalomyelitis Virus (TMEV) is an important model of
multiple sclerosis (MS) (Lipton, 1975). Theiler's virus induces a biphasic disease that,
in the chronic phase, causes a remitting relapsing demyelination of the central nervous
system (CNS) similar to that of MS. Chronic restraint stress in the acute/early phase
profoundly affects vulnerability to TMEV. When an animal is subjected to a stressor,
several biological pathways are activated. Previous studies conducted in our lab have
investigated the role of the hypothalamic pituitary adrenal (HPA) axis and the
glucocorticoid corticosterone. The sympathetic nervous system (SNS) is also activated
by stress and initiates the release of the catecholamine norepinephrine (NE) from the
adrenal medulla. Norepinephrine is believed to have immunosuppressive effects.
Here, we attempt to determine if NE is mediating the deleterious effects restraint stress
on TMEV. To examine this issue, seventy-one 24-day old male CBA mice were
surgically implanted with either a pellet of the β_2-adrenergic antagonist nadolol, or a
pellet containing only vehicle on three days prior to infection. The mice were then
restrained for 12 hours overnight, and infected intracranially with 5×10^4 PFU of the
BeAn strain of TMEV on day 0 post infection. For the following 4 weeks, restraint was
administered 5 nights a week for 12 hours per session. The effects of stress on TMEV
were measured daily using two scales, one to measure the relative sickness of the
animal and the second to measure the degree of hind limb weakness and paralysis.

The body weight of the animals was also recorded daily. This study confirms that norepinephrine is indeed a mediator of the stress response. Animals receiving the placebo had higher mortality, more severe clinical symptoms, and greater hind limb weakness and paralysis than animals receiving the norepinephrine antagonist nadolol.

ACKNOWLEDGEMENTS

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1. Introduction

A compelling body of evidence supports the idea that the central nervous system (CNS) and the immune system are able to communicate. This communication allows environmental stimuli such as physical and psychosocial stressors to effectively modulate the immune system. There are two main conduits through which the brain is able to communicate with the immune system: the hypothalamic pituitary adrenal (HPA) axis and the sympathetic division of the autonomic nervous system. External stressors are processed by the cerebral cortex and activate the paraventricular nucleus of the hypothalamus which secretes corticotropin releasing hormone (CRH). CRH travels to the adenohypophysis (anterior pituitary) via the hypophyseal portal system where it stimulates release of adrenocorticotropic hormone (ACTH) into the blood stream. Circulating ACTH targets the zona glomerulosa of the adrenal cortex resulting in the release of glucocorticoids, which then directly influence the activity of immune effector cells.

External stress stimuli also activate the sympathetic nervous system (SNS) resulting in the release of catecholamines. The SNS originates in the locus ceruleus of the brain stem, from these nuclei, preganglionic efferent fibers run down the spinal cord and exit the central nervous system (CNS) via the thoracic and lumber spinal nerves. The fibers of the SNS are noradrenergic fibers in that they act by releasing the catecholamine norepinephrine (NE). The fact that fibers from the SNS innervate all primary and secondary lymphoid organs and individual immune cells have β_2-adrenoreceptors for NE provides the second pathway for CNS mediation of the immune system (Brodde, Engel, Hoyer, Block & Weber, 1981; Fuchs, Campbell & Munson, 1988; Madden, Sanders, & Felton, 1995. Furthermore, it has been shown that the HPA axis and the SNS noradrenergic system seem to participate in mutual positive feedback loop where activation of one system leads to activation of the other (Chrousos & Gold, 1992).

The influence of stress on the immune system is dependant upon the stressor's duration. Acute stress stimulates the immune system by increasing proliferation and activity of immune cells and then redistributing them to the areas where they are needed (Jellinck, Dhabar & McEwen, 1997). In contrast, chronic stress has been found to suppress immune function making the organism more vulnerable to infection. For

over fifty years evidence has suggested that specific glucocorticoids released by the HPA axis in response to stress may mediate this effect. The last two decades have seen the accumulation of evidence linking the SNS and its end products, catecholamines, to immune system modulation (Elenkov, Wilder, Chrousos, & Vizi, 2000). Recent studies have shown chronic stress increases susceptibility to herpes simplex and influenza viruses in mice and that this effect is mediated by both the glucocorticoid, corticosterone, and the catecholamine, norepinephrine (Sheridan, Dobbs, Jung, Chu, Konstantinos, Padgett, & Glaser, 1998). These studies found that restraint stress suppressed immune responses including natural killer (NK) cell activity, the production of the cytokine interleukin (IL-1), lymphodenopathy and mononuclear cell trafficking. Now that it seems clear that stress can act through specific biological pathways to alter the immune response, logical progression would lead one to inquire as to how this suppression effects the pathophysiology of various diseases.

Stress has been implicated as a contributing factor to the onset and progression of several autoimmune diseases in humans, including multiple sclerosis (MS). With an incidence of approximately 1:2000 in the United States, MS is the most prevelent CNS demyelinating disease in the country. The etiology of MS is presently unknown, but one of the most convincing hypotheses proposes that viral infection during adolescence and early adulthood may be a catalyst. Anecdotal reports suggest that major life stressors frequently trigger the development of MS symptoms (Ackerman, Martino, Heyman, Moyna, & Rabin, 1998; Ackerman, Martino, Heyman, Moyna, & Rabin, 1996). Recent studies using standardized assessment of stressful life events propose that psychological stress precedes both the onset and exacerbation of MS symptoms (Warren et al., 1982). Psychological stress can be used to predict subsequent development of brain lesions in patients with chronic remitting, relapsing forms of MS (Mohr, Goodkin, Boudewyn, Huang, Marietta, Cheuk, & Dee, 2000). However, studies investigating the effects of stress on MS are few and whether there is any effect at all remains controversial. For example, one study examined MS patients in Israel during the missile attacks of the 1991 Persian Gulf War and found that the number of relapses during the war and for two months after were significantly lower than during the preceding two years (Nisipeanu, & Korczyn 1992).

Theiler's virus serves as an important animal model of MS. Theiler's virus induces a chronic remitting relapsing demyelination of the central nervous system similar to that of some forms of MS (Lipton, 1975; Fujinami & Zurbriggen, 1989). Because the disease course is so well characterized (Welsh, Blakemore, Tonks, Borrow, & Nash, 1989 for review), experimentation with TMEV provides an excellent means of investigating the role of corticosterone and norepinephrine in modulating the effects of stress on immunity. Theiler's virus is a picornovirus, which in nature causes an acute enteric infection and occasionally paralysis. The virus may be asymptomatic or fatal depending on the susceptibility of a particular strain of mouse and the virulence of the viral strain. The effects of stress on the pathogenesis of Theiler's virus infection in CBA mice were explored by Campbell, Meagher, Sieve, Scott, Storts, Welsh, and Welsh, 2000. The study revealed that chronic restraint stress suppressed the immune system severely exacerbating the symptomtology of TMEV infection in CBA mice. Infected/restrained mice had a higher incidence of clinical symptoms and mortality than the infected/nonstressed mice. Compared to control mice, restraint stressed animals showed a decreased number of circulating lymphocytes, elevated CNS viral titers, and decreased inflammatory cell infiltration into the CNS one week after infection. This evidence indicates that restraint stress had immunosuppressive effects.

Our laboratory further investigated the roles of restraint stress and corticosterone. We first determined that restraint stress did indeed have a deleterious effect on animal subject's resistance to viral infection. In a second experiment, exogenous corticosterone was administered to infected animals in the absence of restraint stress in order to discern whether cort was the physiological mediating component of the deleterious effect of stress on immune function. We found that administration of exogenous corticosterone resulted in almost identical effect on animal subject's resistance to viral infection. However, this effect was delayed by approximately one week compared to the rapid effect of restraint stress (Meagher, Welsh, T., Sieve, Satterlee, & Welsh, J., In Preparation).

The close connection between the HPA axis and the SNS noradrenergic system may explain why administration of exogenous cort resulted in a delayed effect (Koob, 1999; Crousos et al. 1992). The present study seeks to determine if norepinephrine plays an essential role in the stress-induced down regulation of the murine immune

system. This experiment will use the β_2-adrenergic antagonist, nadolol, that was shown by Sheridan and colleagues (1998) to effectively blockade the immune β_2 receptors. The objective of this experiment is to determine whether nadolol will reduce the deleterious effects of restraint stress on the immune response of male CBA mice to infection with TMEV by blocking the effects of norepinephrine.

2. Methods

2.1 EXPERIMENT 1

2.1.1. Subjects

Subjects were 71 three-week old male CBA mice (Harlan Laboratory). The strain was chosen for its intermediate susceptibility to the BeAn strain of Theiler's virus. Twenty-three of the subjects were run in the first trial and forty-eight subjects were run in a second trial. Mice were assigned to cages by weight, counterbalancing to insure even distribution of heavy and light mice. Mice were housed four per cage in the first trial and three per cage in the second trial. Other than this small difference in housing, identical procedures were used in each trial. The animals were maintained on a 12-hour light/dark cycle. Food and water was available ad libitum. To habituate the mice to human contact, all mice were handled for a couple of minutes each day for three days prior to the behavioral stress procedures.

2.1.2. Acute Clinical and Paralysis Signs.

Following one week of acclimation to the laboratory and handling, each animal was examined daily for the development of clinical signs indicative of acute neurological disease. For each mouse, body weight, clinical score, and paralysis score were recorded daily. The clinical score was based on a numerical scale ranging from 0.00 to 4.00 that increased by 0.25 increments. A score of 0.00 indicates a healthy animal while a score of 3.00 to 3.75 indicates severe neurological symptoms and a score of 4.00 represents death. Scores were determined by assigning point values to clinical signs of illness based upon a standardized subjective system. Interrater reliability was demonstrated by all investigators scoring the animals ($r = 0.95$). Subjects received one quarter point for slightly ruffled fur, and partial grooming. They received additional quarter points for exhibiting each of these symptoms to a greater degree (i.e. prominently ruffled fur and extremely poor grooming). Animals received full points for displaying hunched posture of varying degrees. Other factors that were considered were degree of

lethargy, sunken or crusted eyes, vocalization to touch, and startle to touch. The paralysis score was based on a numerical scale ranging from 0.00 to 5.00. Scores were assigned to each hind leg using a standardized scoring system that considered hind leg weakness, paralysis, and abnormality of gait. A score of 0.00 indicates a normal, strong and functional limb while a score of 5.00 represents a very weak completely paralyzed limb. Interrater reliability was demonstrated for the paralysis scale (r = 0.95). Baseline measures of body weight, clinical and paralysis scores were taken daily during the week prior to stress and infection.

2.1.3. Surgical Implantation of Drug Pellet

After five days of acclimatization to their new environment, cages were randomly assigned to one of four experimental conditions: nadolol/restraint, vehicle/restraint, nadolol/control, and vehicle/control. The animals were anesthetized with a mixture of xylazineand ketamine in lactated ringer's solution. One mL of a stock solution consisting of 1 mL xylazine (100 mg/mL) and 10 mL of ketamine (100gm/mL) was diluted with 4 mL of sterile lactated ringer's to make the anesthesia. Animals received a dose of 0.006 mL per gram of body weight. Anesthetized animals were shaved on the back of the neck with an electric clippers and a 0.5 cm vertical incision was made just below the nape of the neck. The appropriate drug or vehicle pellet was then implanted under the dermis between the neck and right shoulder. The incision was closed with one Michele clip and the animals were placed in heated cages for one hour to recover from the anesthesia. Michele clips were removed two days post surgery.

2.1.4. Behavioral Stress Conditions.

One week after arrival and one day prior to infection, (day -1 post-infection (pi)), mice assigned to the restraint stress condition were placed in well-ventilated 60 mL plastic tubes for 12 hours (8 p.m. to 8 a.m.) five nights per week for four consecutive weeks. Inside the tube, the mouse could move forward and backward freely, but he could not turn around nor did he have access to food or water. Mice in the control condition remained undisturbed in their home cages.

2.1.5. Infection

After the first day of restraint, day 0 pi, all of the mice in the stress and control conditions were anesthetized with isofluorine and infected intracranially with 5×10^4 p.f.u. contained in 20 µl of the BeAn strain of Theiler's virus.

2.1.6 Bleeding

After acclimation to handling, mice were bled (150 µl/bleed) on days -5, 9, and 18 pi. Blood was collected by nicking the saphenous vein of the leg. One day prior to the bleed, the leg to be bled was shaved to facilitate blood collection. During the procedure, a topical anesthetic (Emla) was applied to the shaved leg fifteen minutes prior to pricking of the saphenous vein to insure that the animals felt no pain. Mice were bled one at a time under a fume hood less than 10 seconds following removal from the home cage or restraint tube. The order in which the mice were bled was counterbalanced between conditions. On nights of restraint prior to a bleed, mice were stagger-restrained (placed in restraint tubes nine minutes apart) to account for the time it took to bleed each mouse. In this way, each mouse received the same amount of restraint before bleeding. After bleeding, mice were placed in holding cages outside the animal colony until all mice were bled. Blood samples were taken into heparinized tubes, placed on ice, and centrifuged. The serum was stored at -80 C until it will be used for future assays.

2.1.7. Statistical Analyses.

Differences in body weights, clinical, and paralysis scores were analyzed using an analysis of variance (ANOVA), to examine the behavioral stress by drug interaction. Due to the high and rapid onset of mortality in the restraint conditions, and the subsequent loss of subjects, all analyses were run on data from days 0 to 3 post infection.

3. Results

3.1. Body Weights

Subjects were weighed daily to 0.1 gram accuracy to determine if the nadolol and behavioral stress affected weight loss. Restraint stress resulted in significant weight loss, restraint groups mean weight = 17.07, control groups mean weight = 20.04, $F(1,54) = 29.631$, $p < .0001$. The drug condition by itself did not significantly alter weight between groups, $F(1,54) = 3.534$, $p > .05$. As Figure 1 below illustrates, there was a significant stress by drug interaction, $F(1,54) = 62.672$, $p < .05$. Interestingly, the stress by drug interaction was only seen in the control groups with subjects receiving the nadolol pellet weighing more than subjects receiving the placebo

(Stat p < .05). The between conditions interaction was not significant in the restraint groups. It is possible that the restraint stress overpowered the drug effect.

Figure 1

Body Weights

Drug

■ Nadolol
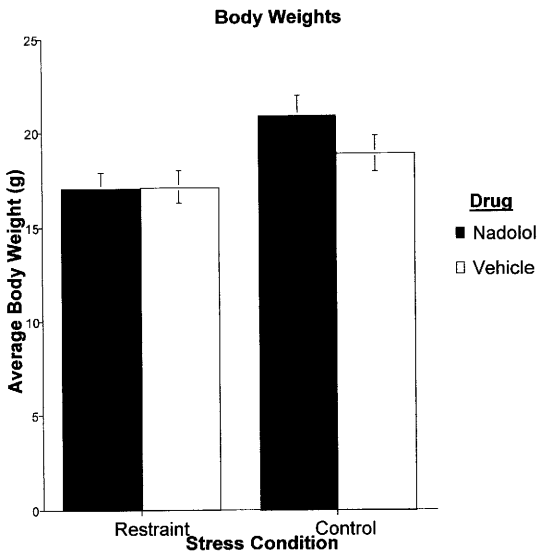
☐ Vehicle

3.2. *Mortality*

Figure 2 depicts survival curves for each of the four conditions. By day three post infection, the vehicle/restrained group began to experience severe mortality. At day ten pi the vehicle/restrained groups had suffered 67% mortality compared to less than 40% for the nadolol/restrained groups. No mice died in the control (no stress)

conditions. It appears as though nadolol delayed both the onset and extent of mortality for the restraint stressed condition.

Figure 2

Percent Survival Over Time
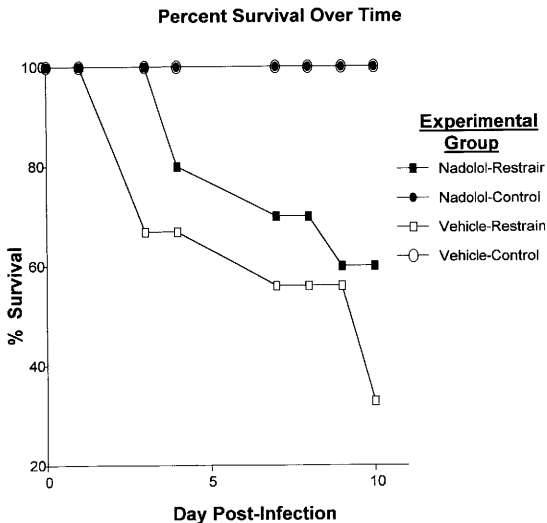
3.3. *Clinical Scores*

Every day, all subjects were examined for symptoms of neurological disease (ruffled fur, poor grooming, hunching, lethargy, and sunken eyes). Increasing clinical scores represent worsening of these symptoms. A one within, two between Analysis of Variance (ANOVA) showed that restraint stress produced more severe clinical

symptoms, $F (1,59) = 142.831$, $p < .0001$, and that administration of the drug nadolol resulted in less severe symptoms, $F (1,59) = 7.552$, $p < .0079$. As Figure 3 demonstrates, the interaction between stress and drug was also significant, $F (1, 59) = 5.687$, $p < .0203$.

Figure 3
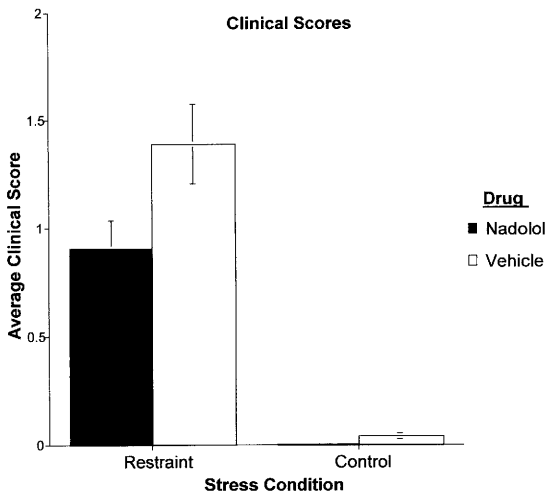
Animals receiving the β_2-adrenergic antagonist, nadolol, had less severe symptoms of neurological disease than animals receiving placebo in both the restraint and control conditions.

3.4. Paralysis Scores

Subjects were scored daily for hind limb weakness and paralysis. An ANOVA confirmed that restraint stress produced more severe clinical symptoms, $F_{(1,58)} = 47.342$, $p < .0001$. Administration of the drug nadolol by itself did not yield a significant decrease in symptom severity, $F_{(1,58)} = 3.538$, $p > .05$, however, as shown in Figure 4 below, there was a significant stress by drug interaction confirming that nadolol administration did reduce the effects of restraint stress on symptoms of neurological disease compared to placebo, $F_{(1,58)} = 5.925$, $p < .0180$.

Figure 4

Clinical Paralysis Scores
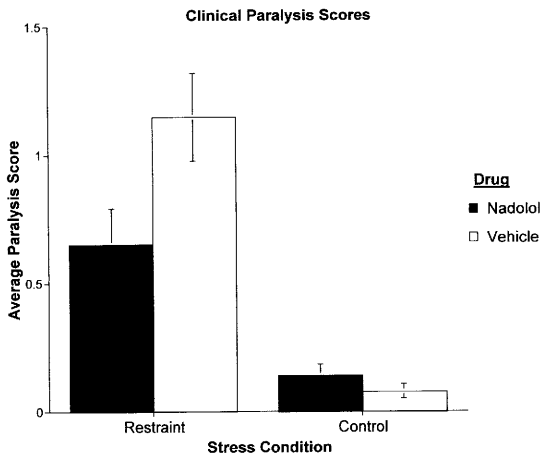
4. Conclusions

The results of this investigation show that the effects of restraint stress on the pathogenesis of TMEV in male CBA mice are at least partly mediated by the

sympathetic nervous system. It was shown that nadolol was successful in reducing the deleterious effects of stress on TMEV infection by decreasing weight loss, reducing both clinical and paralysis symptomtology of neurological disease, and decreasing mortality in restrained mice. There are several possible explanations as to why blockage of β_2-adrenergic receptors might decrease stress-induced down regulation of the immune system.

In acute TMEV infection, natural killer (NK) cells and cytotoxic T (CD8+) cells play a crucial role in the immune response (Welsh et al., 1989). With the exception of T helper 2 (T_{h2}) cells, all immune cells present β_2-adrenergic receptors to varying degrees. Recent evidence suggests that NK and T cells bear the greatest number of β_2-receptors (Van Tits, Michel, Grosse-Wilde, Happel, Eigler, Soliman, & Brodde, (1990); Maisel, Harris, Rearden, & Michel, (1990). It has also been shown that occupation of β_2-receptors by an agonist such as NE elevates intracellular levels of cyclic adenosine monophosphate (cAMP) which is associated with reduced NK and T cell response (Katz, Zaytoun, & Fauci, (1982). Thus, it appears that through modulation of cAMP levels, NE may functionally mediate immune activity. Studies by Hellstrand, Hermodsson, & Stannegard, (1985) serve to further strengthen the role of NE in immune suppression. Hellstrand et al. found that addition of catecholamines to immune cells in vitro decreased NK cell lytic activity. Catecholamines also inhibit both the the phagocytotic ability and the ability to undergo respiratory burst of neutrophils (Zurier, Weissmann, Hoffstein, Kammerman, & Tai 1974).

In addition to the effects that NE appears to have on individual immune cells, it also appears to have a generalized effect on cellular immune trafficking. Cytokines are hormonelike glycoproteins secreted by some immune cells. Cytokines serve as chemical mediators, or immunotransmitters, that serve to regulate the immune response. T helper 1 (T_{h1}) cells secrete several pro-imflammotory, or type 1, cytokines including tumor necrosis factor β (TNF-β), interleukin 2 (IL-2), and interferon γ (INF-γ) while T_{h2} cells secrete the anti-inflammatory, or type 2, cytokines IL-4, IL-10, and IL-13(Abbas, Murphy & Sher, 1996). Importantly, type 1 and type 2 cytokines are mutually inhibitory. Not only do type 2 cytokines inhibit macrophage activation, and T cell proliferation, but they also inhibit the production of pro-inflammatory type 1 cytokines (Abbas et al., 1996; Fearon & Locksley, 1996; Mosmann & Sad, 1996). Even more

importantly, NE appears to potently inhibit T_{h1} activity including type 1 cytokines while simultaneously enhancing T_{h2} and type 2 cytokine activity (Elenkov, Papanicolaou, Wilder, & Chrousos, 1996; Panina-Bordignon, Mazzeo, Lucia, D'Ambrosio, Land, Fabbri, Self & Sinigaglia, 1997). Norepinephrine alters immune response by regulating levels of pro and anti-inflammatory cytokines. The evidence seems strong that NE has a potent immuno-suppressive effect on proliferation and trafficking of immune cells.

This study supports previous findings that chronic restraint stress increases susceptibility to the acute phase of Theiler's virus (Campbell et al., 2000, Meagher et al., In Preparation). In our previous study, we found that administration of exogenous corticosterone mimicked the effects of restraint stress on TMEV infection with a one-week delay. In the present study, significantly high mortality was seen by day 3 post infection. This would seem to support our hypothesis that both the HPA axis and the SNS adrenergic system are instrumental in mediating the effects of restraint stress on the immune response. Corticosterone by itself produced effects more slowly than restraint stress while NE appears to have a more rapid onset. The next step is to investigate whether administration of exogenous NE, or another ß-adrenergic agonist in the absence of restraint stress yields results similar to the present study. If that investigation is successful, it would be most interesting to see if administration of both cort and NE could exactly replicate the effects of restraint stress on immunity.

Experimentation using animal models of MS reveals that the neuroendocrine mechanisms, by which stress may affect the development of the disease, are complex. Theiler's virus provides an illustrative model with which these mechanisms may be experimentally manipulated. Theiler's virus will continue to serve as an important model of MS due to the current hypothesis that a viral infection early in life may initiate a secondary autoimmune response targeting myelin in the CNS. If this hypothesis proves to be correct, TMEV will prove to be an even more crucial model as it promotes investigation of the acute phase of infection, which may operate similarly to the initial viral pathogen involved in multiple sclerosis.

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