DEVELOPING METHODS FOR ANTAGONIZING TNF-α AND IL-1β IN THE CENTRAL NERVOUS SYSTEM

A Senior Honors Thesis

by

ELIZABETH ASHLEY HARDIN

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

April 2006

Major: Biology/Genetics

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Approved as to style and content by:

Mary Meagher (Fellows Advisor)	Edward A. Funkhouser (Executive Director)

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ABSTRACT

Developing Methods for Antagonizing TNF-α and IL-1β in the Central Nervous System

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Stressful life events have been linked to the onset, susceptibility, and even progression of neurodegenerative diseases such as multiple sclerosis (MS). Theiler's murine encephalomyelitis virus (TMEV) infection, a well-characterized animal model of MS, is used in our laboratory to investigate the interaction between social stressors and disease development. Social disruption (SDR), a model of social stress used in our laboratory, appears to worsen Theiler's virus infection through excessive inflammation. Prior findings from our laboratory indicate that pro-inflammatory cytokine IL-6 is partially mediating the negative effects of SDR in the development of Theiler's virus infection. In order to examine the role of other pro-inflammatory cytokines, our objective was to develop techniques to block the cytokines TNF-α and IL-1β. Prior studies have indicated stress-induced release of these cytokines (TNF-α and IL-1β) may mediate the adverse effects of disease development in subsequent immune challenges. Balb/cJ mice were implanted with a permanent indwelling cannula in

the left lateral ventricle of the brain and allowed to recover for one week prior to manipulations. Once the animals recovered from cannulation surgery, neutralizing antibody to TNF-α or IL-1β was administered during the period of SDR. Antibody-SDR treatments continued for one week. Mice were sacrificed the morning following last day of SDR. Brains and sera were collected to measure TNF-α or IL-1β levels. Spleens were harvested to examine the development of glucocorticoid resistance (GCR), a hallmark of SDR, in the TNF-α study only. The ELISA assay was not sensitive enough to the tissue levels of TNF-α, therefore successful antagonism was undetectable. In contrast, IL-1β was elevated during SDR; however, it appears that the antibody was only partially effective at the dose administered. The GCR assay indicated that resistance occurred in antibody treated and control mice in the TNF-α study, signifying that antibody treatment does not interfere with the development of normal social stress effects. Future studies are necessary to identify an effective blocking dose for the neutralizing antibody to IL-1β. In addition, we also need to develop alternative assays, such as RT-PCR or an RNase protection assay, that are sensitive to the levels of TNF-α associated with SDR.

ACKNOWLEDGEMENTS

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I INTRODUCTION

Numerous studies have established that chronic stress suppresses the ability of the immune system to respond to challenge and, thereby, increases vulnerability to opportunistic infection (Ader et al., 1991; Kiecolt-Glaser et al., 1995). Prior research also indicates that the onset, development, and even severity of neurodegenerative diseases are stress-related (Rosch, 1979; McGeer & McGeer, 1995; Njenga et al., 1997; Busciglio et al., 1998; Floyd, 1999; Nguyen et al., 2002). Additionally, stressful life events and poor social support appear to play a role in the onset and progression of autoimmune diseases, such as rheumatoid arthritis (Curtis et al., 2005; Straub et al., 2005; Veldhuijzen van Zanten et al., 2005) and multiple sclerosis (MS) (Ackerman et al., 1998; Mohr et al., 2000). Our laboratory has been using Theiler's murine encephalomyelitis virus (TMEV) infection, an animal model of MS, to better understand the mechanisms that underlie the effects of stress in neurodegenerative diseases, such as MS. Developing techniques to block cytokines will aid in our ability to determine these mechanisms.

Theiler's virus infection is a well-characterized animal model of MS that allows us to investigate central nervous system (CNS) inflammation as it relates to stress (Lipton, 1975; Oleszak et al., 2004). Theiler's virus infection induces a biphasic disease in susceptible strains of mice, such as the Balb/cJ strain used in these experiments. The early acute phase of the disease is primarily CNS inflammatory and is characterized by the infection of central neurons and glia (astrocytes, microglia, and oligodendrocytes). The chronic phase, which serves

as a model for MS, is primarily neuroinflammatory and demyelinating. In order to develop the chronic phase of Theiler's virus infection, the virus must remain in the CNS beyond the resolution of the acute phase (Aubert et al., 1987; Oleszak et al., 2004). The chronic phase is characterized by massive mononuclear inflammatory infiltration, demyelinating lesions, and behavioral manifestations similar to MS (Lipton, 1975; Njenga et al., 1997; Oleszak et al., 2004).

Previous studies in our laboratory investigated the impact of stress, restraint (RST) or social disruption (SDR), on the disease course of Theiler's virus infection. RST involves restraining mice in plastic syringes for a designated period of time (Sheridan et al., 1991; Campbell et al., 2001), whereas SDR involves introducing an intruder mouse into a cage of resident mice for a designated period of time (Avitsur et al., 2001; Stark et al., 2001). These studies have demonstrated that stress exacerbates disease development in Theiler's virus infection in both the acute and chronic phases of the disease (Johnson et al., 2004; Johnson et al., in press; Sieve et al., 2004).

RST led to decreased CNS inflammation during acute infection, and increased behavioral manifestations of encephalitis, mortality rates, and viral titers (Campbell et al., 2001; Sieve et al., 2004). The association between RST and decreased inflammation is further supported by studies using LPS (a bacterial endotoxin) induced endotoxemia, which also found that RST led to decreased inflammation (Padgett et al., 1998; Konstantinos et al., 2001; Quan et al., 2001). In contrast to RST, SDR leads to an increase in systemic

inflammation in mice exposed to LPS (Quan et al., 2001), as well as central inflammation in Theiler's virus infection (Johnson et al., 2004).

Although RST and SDR cause divergent effects on inflammation, both stressors lead to exacerbations of Theiler's virus infection. Mice exposed to SDR developed a more severe disease course, greater inflammation, and were unable to clear Theiler's virus from the CNS over time (Johnson et al., 2004). As mentioned previously, the inability to clear the virus over time is necessary in developing chronic phase illness. SDR also led to the development of glucocorticoid resistance (GCR), or the failure of immune cells to down-regulate in the presence of glucocorticoids (GC), whereas RST typically does not lead to GCR (Johnson et al., 2004; Avitsur et al., 2002; Quan et al., 2001).

GCR develops in the macrophages of the spleen due to SDR, and leads to a dysregulation of pro-inflammatory cytokine function (Avitsur et al., 2002). One function of corticosteroids is to mediate the innate immune response by suppressing the production and proliferation of pro-inflammatory cytokines (IL-6, IL-1β, and TNF-α) and enhancing the action of anti-inflammatory cytokines (IL-10 and IL-4) (Angeli et al., 1999; Lew et al., 1988; Franchimont et al., 1999; Hart et al., 1990). However, in SDR mice, increased inflammation and decreased viral clearance are found even though corticosteroid levels are elevated (Johnson et al., 2004). These findings imply that the development of GCR may exacerbate the inflammatory response due to an immune challenge. The development of GCR (and commensurate dysregulation of pro-inflammatory cytokines) in SDR mice may then be mediating the differential development of inflammation when

comparing RST and SDR (Quan et al., 2001; Johnson et al., 2004; Sieve et al., 2004).

However, pro-inflammatory cytokines may also mediate the development of GCR. For example, elevated levels of IL-6 are associated with an increase in the expression of the beta subtype of the GC receptor (and down-regulation of the alpha subtype receptor). The GC receptor subtype beta has been associated with the development of GCR (Necela et al., 2004). Thus, elevated pro-inflammatory cytokines may be related to the development of GCR, while GCR also results in the production of elevated pro-inflammatory cytokines. The exacerbated inflammatory response associated with the development of GCR may then mediate previously noted impaired clearance of the virus, and other disease exacerbations (Johnson et al., 2004).

The SDR-induced exacerbated inflammatory response with Theiler's virus infection is not only associated with high circulating levels of corticosteroids and GCR (Quan et al., 2001; Johnson et al., 2004), but also elevated cytokine levels (Johnson et al., in press). Others have shown that elevated levels of pro-inflammatory cytokines are associated with an activation of the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system in response to stress (Watkins et al., 1998). Activation of the HPA-axis ultimately results in the release of GC from the adrenal glands, and the sympathetic nervous system releases catecholamines (norepinephrine and epinephrine) that control the activity of various immune organs such as the spleen and thymus (Felten et al., 1998; Watkins et al., 1998).

Cytokines released in the brain and in the periphery serve to mediate the development of inflammatory reactions to stress or immune challenges (Watkins et al., 1998). Cytokine mediated responses to stress are very similar to those seen with an immune challenge alone, such as viral infection or exposure to LPS (Dantzer et al., 1999; Watkins et al., 1998). For example, cytokines alone have been found to cause weight loss, anorexia, decreased motor activity, hypersomnia, reduced interest in social activity, and anhedonia (or the loss of interest in normally pleasurable activities) (Barak, 2002a, 2002b; Pollack et al., 2000). Of interest to the current studies, the levels of pro-inflammatory cytokines (IL-6, IL-1β and TNF-α) are elevated in both the acute phase of Theiler's virus infection (for a review see Oleszak et al., 2004), and separately in SDR (Avitsur et al., 2005; Stark et al., 2002).

Because there is a correlation between stress-induced pro-inflammatory cytokine levels, the development of GCR, and worsening of Theiler's virus disease development, our laboratory investigated the effect of blocking IL-6 on the development of GCR. Based on the relationship between GCR and IL-6 (Necela et al., 2004), it was postulated that IL-6 may induce GCR, and that blocking IL-6 would restore a normal response to GC in mice. Then, normalization of GC sensitivity would lead to a less severe disease course. Antagonism of IL-6 did not affect the development of GCR. However, the negative effects of SDR-induced Theiler's virus infection were reversed (Johnson et al., in submission). Therefore, IL-6 seems to be acting through a mechanism independent of this phenomenon. The idea that stress-mediated inflammation of

the CNS and exacerbation of disease course may be caused by cytokine activation in response to stress is intriguing, and is the focus of ongoing work in our laboratory.

The present studies seek to further examine the immunological mechanisms whereby stress has an effect on the disease course of subsequent immune challenges, and is the first of several subsequent studies. In order to begin to elucidate the role of cytokines other than IL-6, we must first determine methods to block their action, as well as measure the effects of such antagonism. This study is necessary in that it serves to evaluate whether or not SDR results in subsequent increases in pro-inflammatory cytokines TNF-α or IL-1β, and whether or not techniques used previously in our laboratory are effective in blocking these elevated levels. The current studies will effectively lay the groundwork for future studies to investigate the action of these cytokines in mediating the adverse effects of stress on Theiler's virus infection.

II GENERAL METHODS

1. Animals

Male Balb/cJ mice were acquired from Jackson Labs (Bar Harbor, ME). Mice arrived from the breeder at post-natal day (pnd) 23 and were weighed and assigned to individual cages. On pnd 24 mice were cannulated (see below). Following cannulation, mice continued to be individually housed for 48 h. On pnd 26, mice were placed in group-housing, 3/cage, counterbalanced by weight. Mice were maintained on a 12-h light/dark cycle (0500-h/ 1700-h) with ad libitum access to food and water, with the exception of the 2 h SDR sessions.

Intruders for the SDR were retired male breeders, 6-to-8 mo of age, housed with sterilized females to increase territoriality and aggressive behavior. The intruders were selected based on latency to attack both peers and adolescents. All intruders consistently attacked peers within 30 s and adolescents within 2 min on 3 separate occasions. 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 (ULACC).

2. Cannulation surgery

Mice were anesthetized with isoflurane gas (2-5%), heads shaved, eyes coated with petroleum jelly, and placed in a mouse adapted stereotaxic device fitted with a mouse nose cone (#51625, #51609, Stoelting, Wood Dale, IL). The isoflurane delivery system was purchased from Vet Equip (#901806, Pleasanton, CA), and the gas recovery system was purchased from Surgivet (model AES,

Waukesha, WI). An incision was made longitudinally along the midline of the skull and the skull was exposed (hemostats were not needed). The periosteum was removed with a sterile cotton swab, and 2% lidocaine was applied to the wound. Bregma was located and noted. Rostral-caudal leveling was accomplished by measuring the vertical position (z plane) of lambda and bregma and ensuring that they were equalized. The skull was swabbed dry, and the cannulation hole was drilled at +1 mm lateral to bregma and -.4 mm rostral to bregma over the left lateral ventricle (based on Paxinos & Franklin). Guide cannulas (33g), purchased from Plastics One (Roanoke, VA, C315GS-2/SPC), were pre-cut to a depth of 1.75 mm, and implanted and secured with cyanoacrylic gel according the manufacturer's suggestion. Mice were then wrapped with bubble wrap to aid in insulation to combat hypothermia induced by anesthesia, and placed back in the home cage that was warmed over a heating pad. Mice were monitored until fully awake and ambulatory. Once ambulatory, bubble wrap was removed, and mice were returned to the animal colony. For pain control post-surgery, mice were provided with water treated with Tylenol (325 mg/2000 mL). To aid in recovery, mice were also provided with food softened with the Tylenol treated water in the cage. Mice were allowed to recover approximately 6 d prior to any further procedures.

3. Social disruption (SDR) stress

For the stressed mice, intruders were introduced into the cage of resident mice at dark cycle onset (1700 h) for a period of 2 h for a total of 6 SDR sessions the week prior to infection, beginning on pnd 30. SDR occurred for 3 consecutive

sessions, then 1 night off, followed by 3 additional consecutive sessions (Avitsur et al., 2001; Stark et al., 2001), in a separate procedure room. SDR sessions were monitored to ensure that the intruder attacked the residents and that the residents demonstrated submissive behaviors. Intruders that did not attack within 10 min of session initiation were replaced, and the session continued for the remaining 2 h.

4. Sacrifice

Mice were sacrificed the morning following the last session of SDR. Each mouse was overdosed with ketamine (100mg/kg)/xylazine (5 mg/kg), and bled from the brachial artery. Spleen, brain, and spinal cord were harvested appropriately and weighed. Brains and spinal cords were flash frozen in liquid nitrogen, and stored at -80 ºC until an ELISA was performed.

5. Corticosteroid sensitivity assay

On the sacrifice day, spleens were harvested to determine sensitivity of splenocytes to GC regulation (as per Stark et al., 2001). Briefly, spleens were placed in ice-cold Hank's balanced salt solution (HBSS), and mashed to obtain a single cell solution. Red blood cells were then lysed (red blood cell lysis, Sigma, St. Louis, MO), followed by a wash of HBSS+10% heat inactivated fetal bovine serum (FBS-HI, Equitech, Kerrville, TX). Viable cells were then counted using trypan blue dye exclusion and re-suspended at 2.5 X 10^6 cells/mL in supplemented RPMI (Sigma)+10% FBS-HI (supplementation: 75% sodium bicarbonate, 10 mM Hepes buffer, 100 U/mL penicillin, 100 μg/mL streptomycin sulfate, 1.5 mM l-glutamine, and .00035% 2-mercaptoethanol). LPS (Sigma #

L6529) in 2% ethanol was added at a concentration of 1 μg/mL for mitogen stimulation. GC resistance was tested by exposing aliquots of each suspension to dilutions of corticosteroid (0-5 μM, Sigma, St. Louis, MO, #C2505) dissolved in 2% ethanol and supplemented RPMI. Cell suspensions were placed in triplicate in flat-bottomed 96-well micro-titer plates in 100 μL aliquots and incubated for 48-72 h at 37°C and 5% CO_2. After incubation, the cell survival assay was performed.

Cell proliferation was assessed following the manufacturer's instructions with the CellTiter 96 Aqueous non-radioactive proliferation assay kit from Promega (Madison, WI). Color changes were quantified by optical density readings at 490 nm from an EMAX ELISA plate reader (Molecular Devices, Sunnyvale, CA). Mean optical density values for the 3 replications for each sample were used, and the percentage of the corticosteroid-exposed cells versus the LPS stimulated cells was determined for statistical analysis.

6. Statistical analyses

Data are presented as mean ± SEM. Analysis of variance (ANOVA) was used to evaluate differences across SDR-antibody and SDR-vehicle conditions. These analyses were followed by *post hoc* mean comparisons using Duncan's New Multiple Range Test.

III EXPERIMENT 1: TNF-α

1. Introduction

Our laboratory has previously demonstrated that pro-inflammatory cytokine IL-6 is necessary in mediating the adverse effects of Theiler's virus infection. However, IL-6 alone is not sufficient in replicating the negative effects of SDR on the development of Theiler's virus infection. This indicates that pro-inflammatory cytokine IL-6 is not solely responsible for the SDR-induced exacerbations of Theiler's virus infection. Theiler's virus infection is not only associated with high transcript levels of pro-inflammatory cytokine IL-6, but also TNF-α, in susceptible strains of mice (Oleszak et al., 2004). High levels of TNF-α are also associated with SDR and challenges to the immune system (Avitsur et al., 2005). Therefore, based on previous experiments, TNF-α may play a significant role in the mechanism mediating the adverse effects of Theiler's virus infection. Before behavior and illness measures can be conducted, the successful blockage of TNF-α in the CNS must be verified. This study investigated whether or not SDR elevated levels of pro-inflammatory cytokine TNF-α and, if so, whether these elevations could be antagonized using the neutralizing antibody technique. Therefore, this study is necessary to begin further investigation into the role of other pro-inflammatory cytokines (TNF-α and IL-1β) in mediating the adverse effects of Theiler's virus infection.

2. Methods

2.1 Procedure

Upon arrival, mice were weighed and caged individually. The next morning, cannulation surgeries occurred. Mice were allowed to recover in individual housing for 2 d. On pnd 27, mice were weighed again and assigned to cages in a counterbalanced manner, based on weight. The Tylenol water was removed and plain water was added to each cage. On pnd 30, antibody or vehicle was administered daily beginning at 1300 h, and then the stressed animals were administered SDR at from 1700-1900 h. Mice were sacrificed the morning following the last session of SDR.

2.2 Antibody to TNF-α

Polyclonal antibody to mouse TNF-α was purchased from R&D Systems (AF-406-NA, Madison, WI). To provide the stock solution, the original 100 μg was dissolved in 1 mL sterile PBS. The stock solution was then further diluted by 1:20 to result in a 5 ng/μL solution.

2.3 Antibody administration

The 5 ng/μL solution of neutralizing antibody was administered using a 25-μL Hamilton syringe and a pump set to administer 60 μL/hour through a 36-g cannula (Plastics One, Roanoke, VA, C315IDC/SPC). 2 μL was then administered over 2 min, followed by a 30 s delay to prevent backwash of the solution before the cannula was removed. The total dose per animal was then 10 ng. This dose was based on the 50% neutralizing dose information provided by the manufacturer. Control animals were administered mouse immunoglobulin

(Ig, Santa Cruz Biotechnology, Inc #SC-2025) in the same volume of sterile saline as the vehicle, in order to account for generalized responses to proteins.

2.4 Efficacy of antibody treatment in blocking TNF-α

In order to assess the efficacy of the antibody dose in blocking TNF-α, 12 mice were used. These animals arrived from breeder, were cannulated, and underwent SDR as described in the general methods section. A 1 (SDR) x 2 (antibody, vehicle) was used. At 1300 h daily, 4 h prior to SDR, antibody or vehicle administration occurred. Following SDR/treatment sessions, all animals were sacrificed and spleens were harvested for GCR assessment, while brain tissue and sera were assessed for TNF-α levels, using an ELISA assay.

2.5 TNF-α ELISA

An ELISA for the TNF-α assay was purchased from R & D Systems (R & D Systems Madison, WI), and sera (frozen at -80°C between sacrifice and the time of the assay) was assessed according to the manufacturer's instructions. Brain tissue was homogenized in 1 mL of Dulbecco's Eagle Medium (Sigma) and flash frozen. Tissue was stored at -80°C until the time of the assay. Tissue was then thawed and centrifuged at 2000 rpm for 5 min. 50 μL of the supernatant per well was used to assess TNF-α levels in the brain.

3. Results

3.1 Arrival Body Weights

Mice were weighed upon arrival at pnd 23, prior to all experimental manipulations, and were counterbalanced and housed in groups of three. Figure

1A shows initial body weights across groups. ANOVA was used to confirm that there were no group differences prior to manipulations, $F(1,10) = .368$, $p > .05$.

3.2 Spleen Weights

Spleens were harvested from mice immediately upon sacrifice and weighed. Figure 1B shows spleen weights in the antibody and vehicle treatment groups. ANOVA confirmed that there were no group differences in these animals, $F(1,10) = .238$, $p > .05$. Splenomegaly is often associated with the development of GCR, and the spleen sizes found here (.077g) are somewhat higher than our laboratory typically finds in non-stressed animals (.069g).

3.3 GCR Assay

Figure 1C shows GCR development with IL-6 antibody treatment at day 7 post infection, from a previous study in our laboratory (top panel, Johnson et al., in submission), and GCR from the current study (bottom panel). The data from the previous study is included in order to illustrate both normal GC sensitivity (NON-AbTx, NON-VEH, and SDR-VEH groups) and GCR (SDR-AbTx). The bottom panel of Figure 1C shows that SDR-antibody treated mice in the TNF-α experiment developed GCR (cell survival rates of 80% or greater, regardless of corticosteroid level). ANOVA confirmed that no significant decrease in cell survival occurred over increasing corticosteroid level, $F(4,32) = .139$, $p > .05$. Therefore, as with antibody to IL-6, antibody treatment to TNF-α does not interfere with the development of GCR.

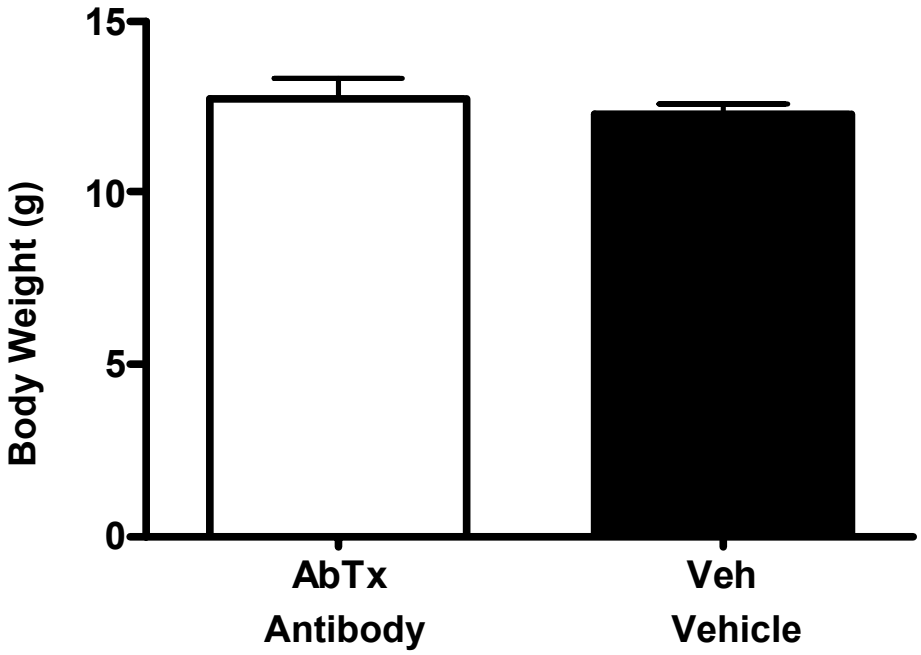

Figure 1A. Arrival body weights for TNF-α mice

Mice arrived at pnd 23 and were weighed prior to all manipulations. No significant differences were found across groups prior to surgery or social disruption ($p>.05$).

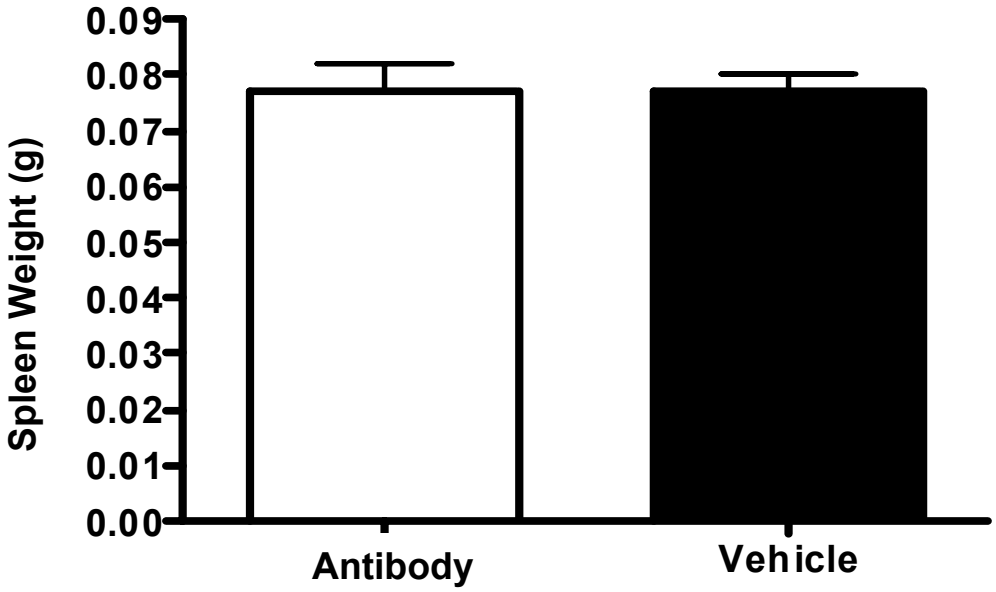

Figure 1B. Spleen weights for TNF-α mice

Spleens were harvested from mice immediately upon sacrifice and weighed. GCR is often highly associated with enlarged spleens. There were no significant differences in spleen weights between antibody and vehicle treatment groups in TNF-α mice ($p>.05$). Therefore, antibody treatment did not interfere with the development of GCR.

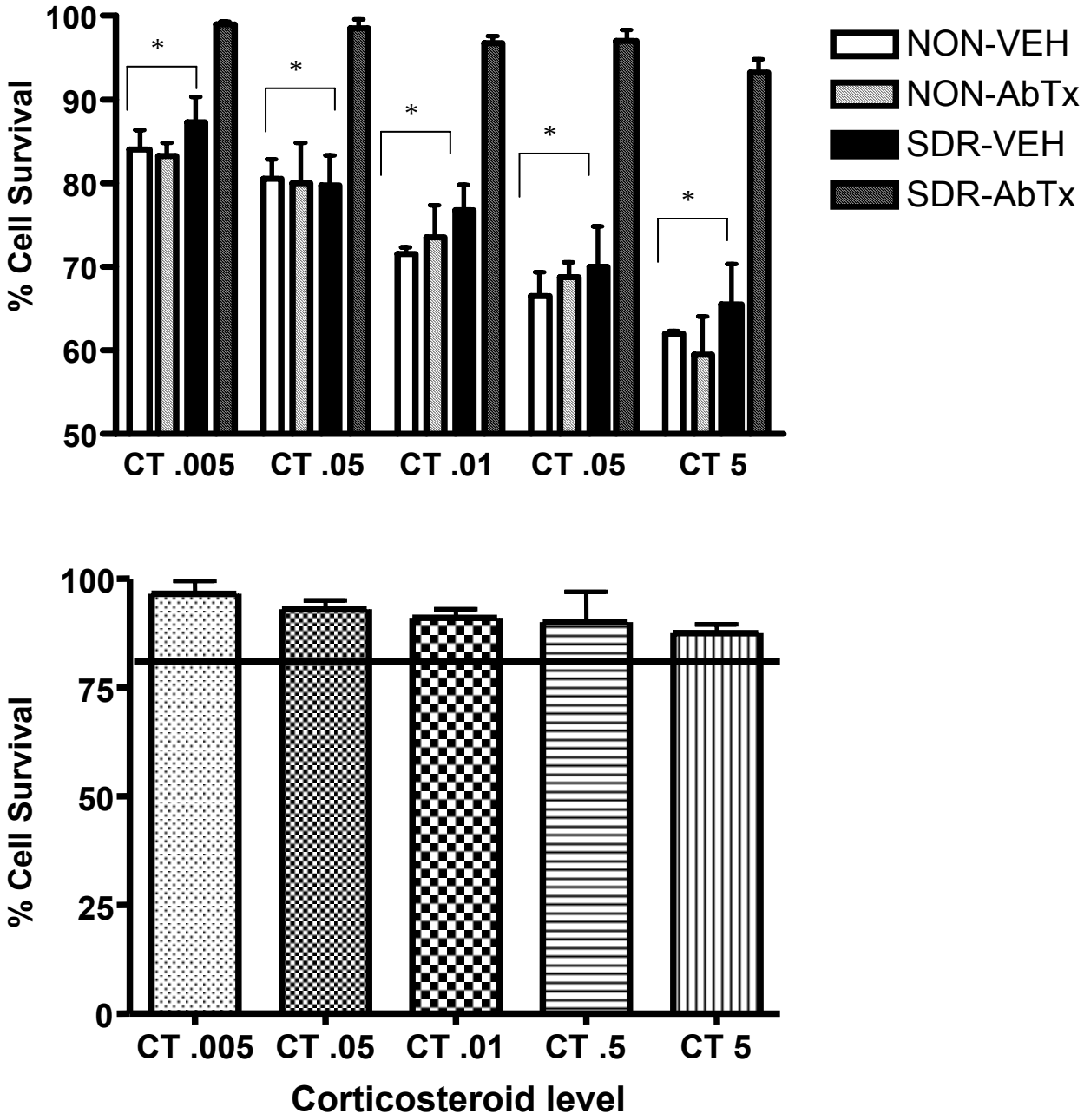

Figure 1C. Glucocorticoid resistance (GCR) with IL-6 (top graph) and TNF-α antibody (bottom graph) treatment

GCR development with IL-6 antibody treatment at day 7 post infection is shown in the graph on the top. Both SDR groups developed GCR prior to infection. Non-SDR mice, in comparison with SDR mice, did not develop GCR, indicated by the low cell survival rate at increasing levels of corticosteroids. SDR-VEH and SDR-AbTx mice both developed glucocorticoid resistance, indicating that the IL-6 antibody treatment did not interfere with this phenomenon. The graph on the bottom shows that TNF-α antibody treatment in SDR-antibody mice does not interfere with the development of GCR

3.4 Verification of antibody treatment

In order to verify that TNF-α was elevated due to SDR in the current study, and that this elevation was blocked by the neutralizing antibody treatment, an ELISA for TNF-α was used to test both brains and sera. The levels of TNF-α were not quantifiable, in either the brain or sera, using an ELISA. Therefore, successful antagonism of TNF-α was not measurable.

4. Discussion

Previous studies in our laboratory investigating pro-inflammatory cytokine IL-6 indicated that SDR alone elevates cytokine levels, and that the neutralizing antibody treatment successfully blocked cytokine levels in both the brain and periphery (Johnson et al., in submission). This study examined whether SDR elevated levels of TNF-α in the brain and periphery, and if so, whether or not these elevations could be antagonized using the neutralizing antibody technique. Splenomegaly and the development of GCR occurred in both antibody and vehicle groups in the TNF-α experiment, indicating successful application of SDR. These findings also show that antibody treatment does not interfere with the development of GCR. An ELISA conducted in this study indicated that levels of TNF-α were not quantifiable in either the CNS or the periphery in mice receiving either antibody or vehicle treatments. Therefore, blockage of the cytokine was not able to be measured. Before we are able to verify whether or not TNF-α can be successfully blocked in the CNS and periphery, and further investigate the role of this cytokine in the mechanisms mediating the adverse

effects on disease course development, more sensitive assays must be developed, such as RT-PCR or using blood plasma.

IV EXPERIMENT 2: IL-1β

1. Introduction

The aim of the second study in this series was to develop methods to adequately block IL-1β, as well as to measure successful antagonism. Similar to TNF-α, IL-1β is also dysregulated by GCR. IL-1β plays an important role in the development of inflammatory responses, both due to stress and infection (Watkins et al., 1998). Thus, the current study will use methods similar to Experiment 1 to develop antagonism methodologies for IL-1β.

2. Methods

2.1 Procedure

Upon arrival, mice were weighed and caged individually. The next morning, cannulation surgeries occurred. Mice were allowed to recover in individual housing for 2 d. On pnd 27, mice were weighed again and assigned to cages in a counterbalanced manner, based on weight. The Tylenol water was removed and plain water was added to each cage. On pnd 30, antibody or vehicle was administered daily beginning at 1300 h, and then the stressed animals were administered SDR at from 1700-1900 h. Mice were sacrificed the morning following the last session of SDR.

2.2 Antibody to IL-1β

Polyclonal antibody to mouse IL-1β was purchased from R&D Systems (AF-406-NA, Madison, WI). To provide the stock solution, the original 100 μg was dissolved in 1 mL sterile PBS. The stock solution was then further diluted by 1:33 to result in a 2 ng/μL solution.

2.3 Antibody administration

The 2 ng/μL solution of neutralizing antibody was administered using a 25-μL Hamilton syringe and a pump set to administer 60 μL/hour through a 36-g cannula (Plastics One, Roanoke, VA, C315IDC/SPC). 2 μL was then administered over 2 min, followed by a 30 s delay to prevent backwash of the solution before the cannula was removed. The total dose per animal was then 10 ng. This dose was based on the 50% neutralizing dose information provided by the manufacturer. Control animals were administered mouse immunoglobulin (Ig, Santa Cruz Biotechnology, Inc #SC-2025) in the same volume of sterile saline as the vehicle, in order to account for generalized responses to proteins.

2.4 Procedure

In order to assess the efficacy of the antibody dose in blocking elevations in IL-1β, 12 mice were used. These animals arrived from breeder, were cannulated, and underwent SDR as described in the general methods section. A 1 (SDR) x 2 (antibody, vehicle) was used. At 1300 h daily, 4 h prior to SDR, antibody or vehicle administration occurred. Following SDR/treatment sessions, all animals were sacrificed and brain tissue and sera were assessed for IL-1β levels, using an ELISA assay.

2.5 IL-1β ELISA

An ELISA for the IL-1β assay was purchased from R & D Systems (R & D Systems Madison, WI), and sera (frozen at -80°C between sacrifice and the time of the assay) was assessed according to the manufacturer's instructions. Brain tissue was homogenized in 1 mL of Dulbecco's Eagle Medium (Sigma) and flash

frozen. Tissue was stored at -80°C until the time of the assay. Tissue was then thawed and centrifuged at 2000 rpm for 5 min. 50 μL of the supernatant per well was used to assess IL-1β levels in the brain.

3. Results

3.1 Arrival Body Weights

Mice were weighed upon arrival at pnd 23, prior to all experimental manipulations, and were counterbalanced and housed in groups of three. Figure 2A shows initial body weights across groups. ANOVA was used to confirm that there were no group differences prior to manipulations, $F(1,10) = .488$, $p > .05$.

3.2 Spleen Weights

Spleens were harvested from mice immediately upon sacrifice and weighed. Figure 2B shows spleen weights in the antibody and vehicle treatment groups. Splenomegaly is often associated with the development of GCR. While animals receiving SDR alone had enlarged spleens compared to the animals receiving both SDR and antibodies, the difference was not significant. ANOVA confirmed that there were no group differences in these animals, $F(1,10) = 2.747$, $p > .05$.

3.3 Verification of antibody treatment

In order to verify that IL-1β was elevated due to SDR in the current study, and that this elevation was blocked by the neutralizing antibody treatment, an ELISA for IL-1β was used to test both brains and sera. The data in Figure 2C indicate that levels of IL-1β were elevated due to SDR. There was a marginal reduction effect only in the CNS of the IL-1β treated mice, $F(1,9) = 2.159$, $p = .1$.

The IL-1β antibody treatment was not successful in blocking IL-1β in the periphery, $F(1,9) = .63$, $p > .05$.

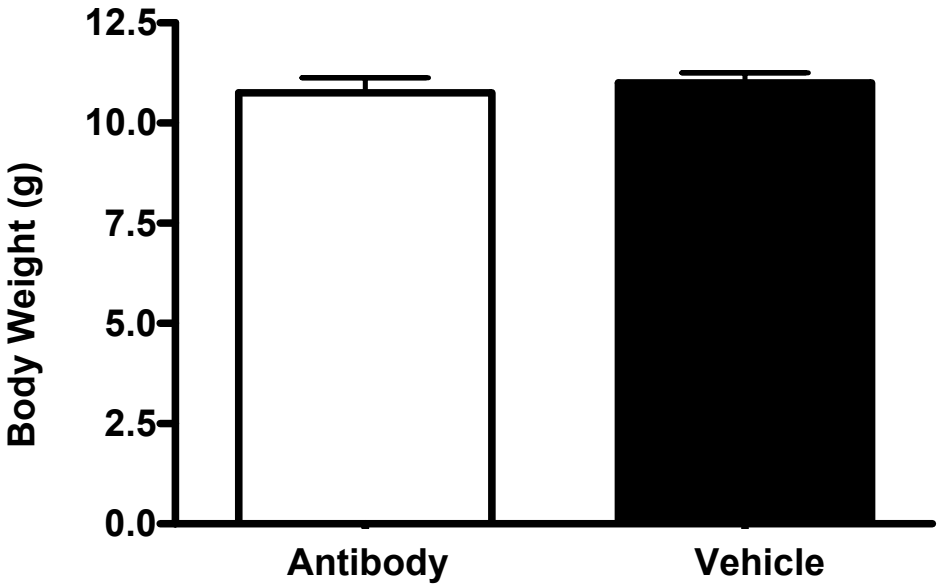

Figure 2A. Arrival body weights for IL-1β mice

Mice arrived at pnd 23 and were weighed prior to all manipulations. These graphs demonstrate that there were no significant differences across groups prior to surgery or social disruption ($p>.05$).

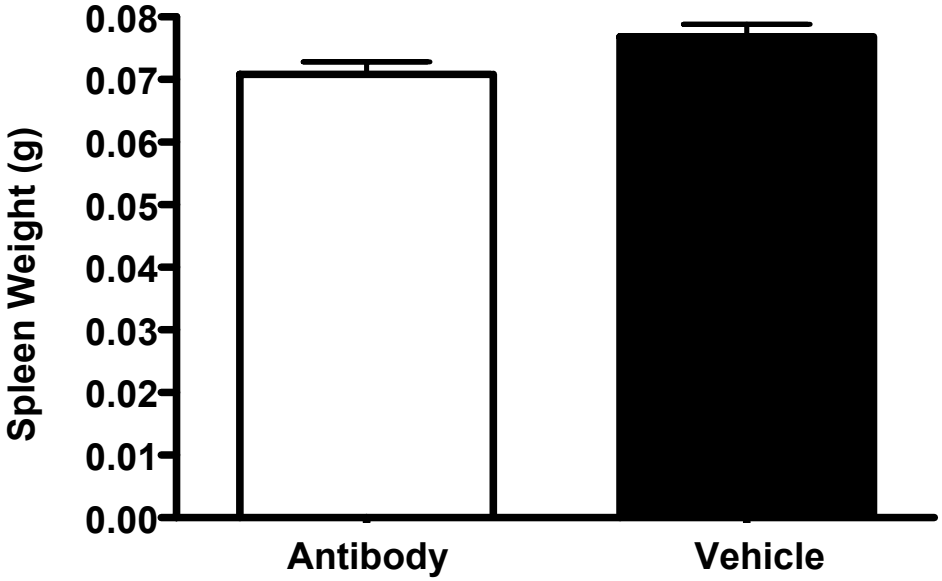

Figure 2B. Spleen weights for IL-1β mice

Spleens were harvested from mice immediately upon sacrifice and weighed. GCR is often highly associated with enlarged spleens. There were no significant differences in spleen weights between antibody and vehicle treatment groups in IL-1β mice ($p>.05$). Therefore, antibody treatment did not interfere with the development of GCR.

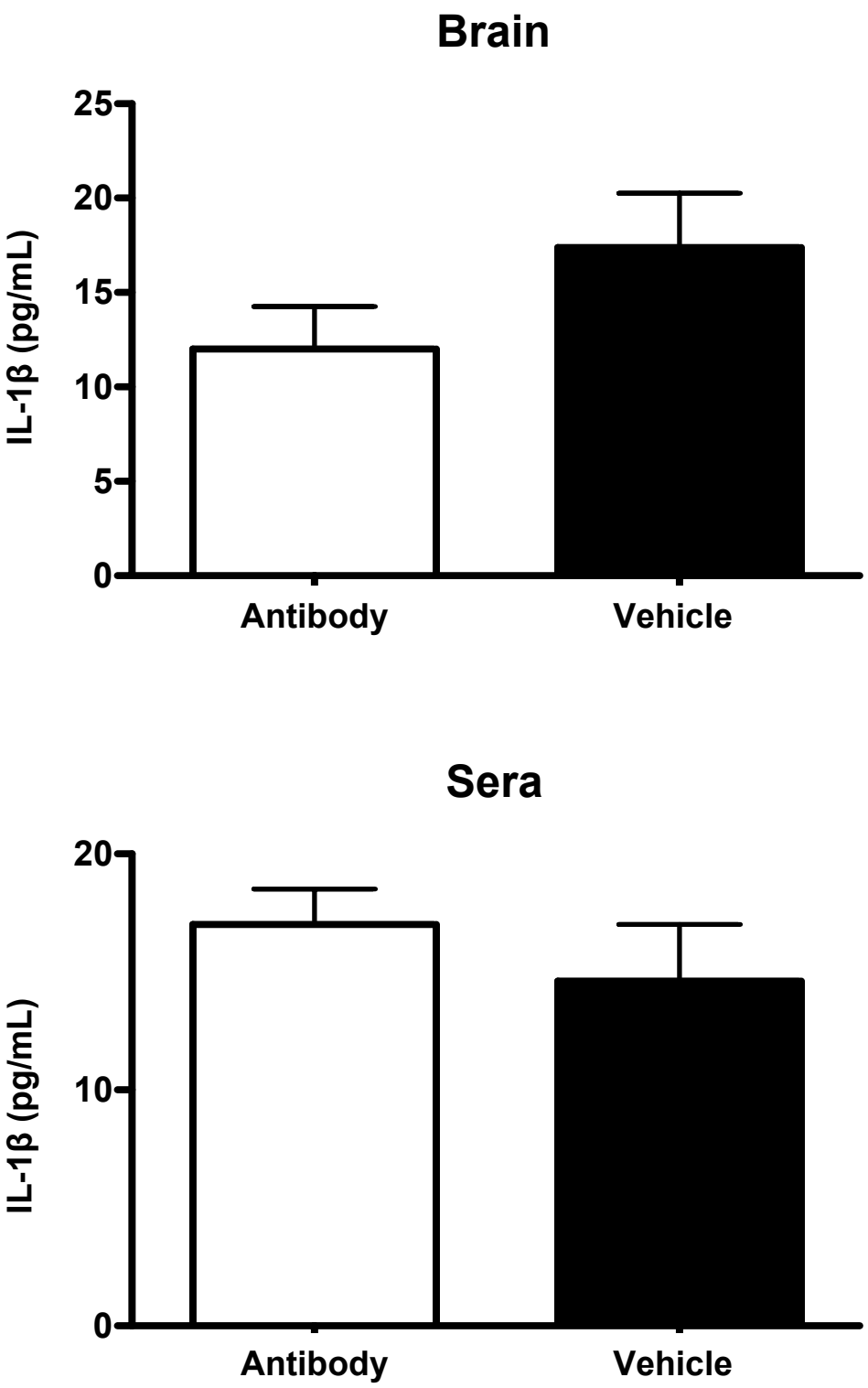

Figure 2C. Efficacy of neutralizing antibody treatment in IL-1β mice

Mice were sacrificed the morning after their final SDR session, and brains and blood sera were collected to analyze the remaining levels of IL-1β using an ELISA. A trend was seen in the reduction of IL-1β in the brain (p=.1).

4. Discussion

This study examined whether IL-1β in the brain and periphery could be antagonized using the neutralizing antibody technique. Splenomegaly was marginal in the vehicle group in the IL-1β experiment, indicating probable successful application of SDR. Thus, the development of GCR is likely in these mice. Although spleen weights were lower (not significantly) in the antibody treated animals, they probably also developed normal GCR, based on our previous findings. Prior studies indicated that GCR developed in socially stressed mice receiving antibody to both IL-6 and TNF-α (Johnson et al., in submission). Based on those prior studies, as well as time and money constraints, we did not perform a GCR assay on IL-1β mice. Unlike the TNF-α study, an ELISA run on IL-1β mice revealed elevated levels of cytokine, and a marginal blocking effect was found in the CNS of antibody treated mice. However, the levels of IL-1β in the periphery were not altered. This indicates that the blocking dose for IL-1β needs to be explored in future studies before the role of IL-1β in the mechanisms mediating the adverse effects of Theiler's virus infection can be further investigated.

V SUMMARY AND CONCLUSIONS

1. Summary

These studies examined techniques used to antagonize pro-inflammatory cytokines TNF-α and IL-1β in the CNS. Previous studies have indicated that the exacerbated effects of SDR on Theiler's virus infection may be due to the action of pro-inflammatory cytokines (IL-6, IL-1β, and TNF-α). Our laboratory previously found IL-6 to be necessary, but not sufficient, in mediating the negative effects of Theiler's virus infection (Johnson et al., in submission). Therefore, IL-6 alone is not responsible for the exacerbations in motor impairment, excessive inflammation, and decreased viral clearance. Therefore, this study began to investigate the roles of other pro-inflammatory cytokines, TNF-α and IL-1β.

The objective of the TNF-α study was to detect whether or not SDR elevated the levels of TNF-α in the CNS and periphery, and if so, whether these elevations could be successfully antagonized using techniques previously developed in our laboratory. Neutralizing polyclonal antibody against murine TNF-α was administered through a permanent, chronic indwelling cannula implanted in the left lateral ventricle of the brain. A GCR assay indicated that antibody treatment did not interfere with the development of GCR. Levels of TNF-α were not quantifiable by an ELISA. An assay that is more sensitive to levels of TNF-α, such as RT-PCR or an RNase protection assay, is needed. Because levels of TNF-α were not detected, successful antagonism of TNF-α is unknown.

The objective of the IL-1β experiment was also to detect whether or not SDR altered the levels of IL-1β in the CNS and periphery, and whether these elevations could be successfully antagonized using techniques previously developed in our laboratory. Neutralizing polyclonal antibody against murine IL-1β was administered through a permanent, chronic indwelling cannula implanted in the left lateral ventricle of the brain. An ELISA indicated that levels of IL-1β were elevated in the CNS and periphery. Partial antagonism in the CNS of IL-1β was indicated by the low, however insignificant, p-value (p=.1). Therefore, a dose response curve is needed in order to block the action of IL-1β, and begin investigating the role of IL-1β in mediating the adverse effects of Theiler's virus infection.

2. General Discussion and Conclusions

These studies showed that the antagonism of pro-inflammatory cytokines TNF-α and IL-1β requires different techniques than those previously used in our laboratory to block the action of IL-6. Previous studies in our laboratory have shown that SDR leads to exacerbations of Theiler's virus infection including elevated motor impairment, elevated inflammation, and decreased viral clearance (Johnson et al., 2004; Johnson et al., in submission). These studies also indicated that SDR led to the development of GCR. As stated previously, GCR is the failure of immune cells to down-regulate in the presence of GC, and results in the dysregulation of pro-inflammatory cytokines. Elevations in inflammation due to SDR may result in the exacerbation of acute Theiler's virus infection.

Prior studies in our laboratory have indicated that antagonism of IL-6, using the methods previously described, led to the reversal of the adverse effects of Theiler's virus infection (Johnson et al., in submission). Antagonism of IL-6 was found to reverse SDR-induced exacerbations of motor impairment, elevated inflammation, and decreased viral clearance (Johnson et al., 2004; Johnson et al., in submission). Although IL-6 is necessary in mediating the adverse effects of Theiler's virus infection, it is not sufficient in mimicking the effects of SDR alone. Therefore, IL-6 is not the only mediator of the SDR-induced adverse effects of Theiler's virus infection. Thus, previously noted elevations in pro-inflammatory cytokine IL-6 led to the hypothesis that other pro-inflammatory cytokines (IL-1β and TNF-α) may also be mediating the adverse effects of Theiler's virus infection.

This study was necessary in order to begin elucidating the roles of pro-inflammatory cytokines TNF-α and IL-1β in mediating the adverse effects of Theiler's virus infection due to stress. Before beginning an expensive, time-consuming study, our laboratory was interested in whether SDR alone elevated levels of pro-inflammatory cytokines TNF-α and IL-1β. Our laboratory was also interested in whether these elevated levels of cytokines could be successfully antagonized by replicating the methodology used in the previous IL-6 study. Since previous studies in our laboratory have shown that cytokine levels of IL-6 were elevated in SDR mice in comparison to control mice (Johnson et al., in submission), control mice were not incorporated into this study. Once techniques have been developed to successfully block elevated levels of TNF-α and IL-1β, a

study analyzing the acute and chronic phase development of Theiler's virus infection involving SDR and control mice will be conducted.

In the TNF-α experiment, the successful application of SDR is indicated by the development of splenomegaly (enlarged spleens highly correlated with SDR), in both antibody and vehicle treated mice, and GCR, a hallmark of SDR. Spleens of antibody and vehicle mice were larger than spleens of mice not experiencing SDR (based on previous spleen weights of control mice in our laboratory). A GCR assay performed on the spleens of TNF-α mice indicated that resistance of macrophages to GC was present. The development of GCR in mice receiving antibody to TNF-α or vehicle treatment implies that this cytokine is not necessary for the development of the insensitivity to this hormone. The inability of an ELISA to detect levels of TNF-α in the CNS or periphery indicates the need to develop more sensitive assays to the cytokine, as stated previously. In addition to an RT-PCR or RNase assay, blood plasma, instead of blood sera, could be used in the ELISA.

The IL-1β experiment also indicated that SDR was successfully applied. The spleens of IL-1β mice were enlarged (above the average weight of spleens from previous control mice in our laboratory). A GCR assay was not performed on IL-1β mice due to data provided by the IL-6 and TNF-α studies that indicated the development of GCR in the presence of neutralizing antibodies. Our laboratory also wanted to ensure successful blockage of IL-1β before spending our resources on a GCR assay. An ELISA detected elevations in cytokine levels in the CNS and periphery of IL-1β mice receiving antibody treatment. However,

the antagonism of IL-1β in the CNS was only partially effective, indicated by the low, but insignificant, p-value obtained (p=.1). Unlike the partial antagonism achieved in the CNS, no blockage of IL-1β was detected in the periphery.

Adjustments to the techniques used in the TNF-α and IL-1β studies are necessary. Once levels of TNF-α are successfully detected, an adequate blocking dose can be found. Future studies will then be able to explore a dose response curve for antibodies to TNF-α. Because levels of IL-1β were marginally blocked in the CNS, a dose-response curve needs to be investigated. The selective homogenization of certain portions of the brain could also be employed, due to the uneven production of cytokines in different regions of the brain. This could provide better insight into the ability of polyclonal antibodies to successfully antagonize IL-1β levels in the periphery.

Once methods to antagonize pro-inflammatory cytokines TNF-α and IL-1β have been developed, future studies in our laboratory will further explore the roles of these cytokines in mediating the adverse effects of SDR on the development of neurodegenerative diseases, such as rheumatoid arthritis and MS. These studies will seek to investigate the necessity and sufficiency of pro-inflammatory cytokines (TNF-α and IL-1β) in the development of the adverse effects of Theiler's virus infection. These studies could provide insight into possible human interventions. MS patients, for example, seem to experience stressful life events prior to the onset of symptoms. In addition, GCR in MS patients has been previously associated with chronic stress (Mohr et al., 2005). These studies and others show the complex interactions of social stressors and

the immune system. Ultimately, our goal is to understand the mechanisms by which social stressors alter immune function.

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