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Title of Thesis: "Role of Cytokines and Neurotrophins in the Central Nervous System in Venezuelan Equine Encephalitis Pathogenesis"

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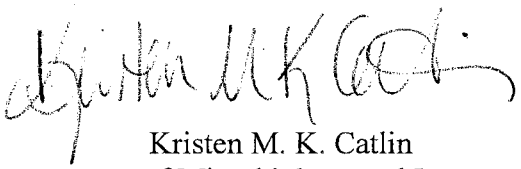

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ABSTRACT

Title of thesis: Role of Cytokines and Neurotrophins in the Central Nervous System in Venezuelan Equine Encephalitis Virus Pathogenesis

Kristen M.K. Catlin, Candidate, Master of Science, 2001

Thesis directed by: Franziska B. Grieder, Ph.D., D.V.M., Assistant Professor, Department of Microbiology and Immunology and Eleanor S. Metcalf, Ph.D., Professor, Department of Microbiology and Immunology

Venezuelan equine encephalitis virus (VEE) is a mosquito-borne alphavirus capable of causing acute febrile illness and encephalitis in man. Currently, there is no licensed vaccine to combat this human health threat and no treatment other than supportive therapy. The inflammatory immune response in the central nervous system (CNS) has been implicated as a contributing factor in a number of neurodegenerative disorders and encephalitic virus infections. The studies detailed here have attempted to elucidate the role of the cytokine and neurotrophin response in the CNS in response to VEE infection by characterizing the gene and protein expression of a number of proinflammatory and anti-inflammatory cytokines in primary astrocyte cultures as well as in the CNS of mice following infection with VEE strains of differing degrees of neurovirulence. Reverse transcription-polymerase chain reaction, RNase protection assays, and enzyme-linked-immunosorbent assays were used to achieve this. Data revealed proinflammatory cytokines were significantly upregulated following infection with the virulent VEE, while neurotrophins were significantly upregulated following infection with the attenuated VEE. These findings may contribute to the goal of manipulating these immune responses for therapeutic benefit.

ROLE OF CYTOKINES AND NEUROTROPHINS IN THE CENTRAL NERVOUS SYSTEM IN VENEZUELAN EQUINE ENCEPHALITIS VIRUS PATHOGENESIS

By

Kristen M. K. Catlin

Thesis submitted to the Faculty of the
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DEDICATION

To Franziska, who taught me to persevere.

To my parents, Diana and Gary, who have always encouraged me to be my best.

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LIST OF ABBREVIATIONS

AD	Alzheimer s Disease
AIDS	Acquired Immune Deficiency Syndrome
ALS	Amylotrophic Lateral Scleropathy
BDNF	Brain derived neurotrophic factor
BDV	Borna Disease Virus
CNTF	Ciliary neurotrophic factor
EAE	Experimental Autoimmune Encephalomyelitis
EAN	Experimental Autoimmune Neuropathy
ELISA	Enzyme linked immunosorbent assay
GBS	Guellain Barre Syndrome
GDNF	Glial cell-line derived neurotrophic factor
HAM	HTLV-Associated Myelopathy
HD	Huntington s Disease
HIV	Human Immunodeficiency Virus
HTLV	Human T-Lymphotrophic Virus
IFN	Interferon
IL	Interleukin
iNOS	Inducible nitric oxide synthase
MOI	Multiplicity of infection
MS	Multiple Sclerosis
NGF	Nerve growth factor
NO	Nitric oxide
NSV	Neurovirulent Sindbis Virus strain
NT	Neurotrophin
PCR	Polymerase chain reaction
PD	Parkinson s Disease
PFU	Plaque forming unit
RPA	RNase protection assay
RT	Reverse transcription
SCID	Severe combined immunodeficiency
SFV	Semliki Forest Virus
SV	Sindbis Virus
TE12	Attenuated Sindbis Virus strain
TGF	Transforming growth factor
TNF	Tumor necrosis factor
TSP	Tropic Spastic Paraparesis
VEE	Venezuelan Equine Encephalitis Virus

INTRODUCTION

Introduction to Cytokines and Neurotrophic Factors

Cytokines are small polypeptides or glycoproteins possessing many different and far-reaching effects in the body and are an essential component of the immune system. These molecules are usually activated and secreted in response to trauma, illness, or infection (12, 64). Certain cytokines, including interleukin (IL)-1, tumor necrosis factor-alpha (TNF-α), IL-6, interferon-alpha (IFN-α), -beta (ß), and -gamma (γ), and IL-12, have pro-inflammatory functions and effects, while others, including IL-2, IL-10, and transforming growth factor-beta (TGF-ß), display anti-inflammatory effects. The production and secretion of cytokines must be tightly regulated or, despite their ability to defend against injury and microbial invaders, these potent molecules may also cause host tissue destruction in response to infection and/or trauma (54, 124, 139). Recent studies have implicated pro-inflammatory cytokines in the pathogenesis of a variety of human diseases including, but not limited to, Crohn s disease (63), atherosclerotic plaques (47), autoimmune encephalomyelitis (96), Japanese encephalitis (132), and Venezuelan equine encephalitis (124, 126).

A small subset of the cytokine superfamily, the neurotrophins, provide support and protection for neurons and other cell types of the Central Nervous System (CNS), including glial cells. Neurotrophins enhance neuronal differentiation, induce proliferation, influence electrical synapse activity, and protect cells of the peripheral and central nervous systems against apoptosis. Known neurotrophins include Brain Derived Neurotrophic Factor (BDNF), Nerve Growth Factor (NGF), Neurotrophin-3 (NT-3),

Neurotrophin-4 (NT-4), Ciliary Neurotrophic Factor (CNTF), and Glial Cell Line Derived Neurotrophic Factor (GDNF) (64). Because neurotrophins affect functional activities and survival of neurons and glial cells, current investigations are attempting to elucidate the viability of neurotrophins as therapeutic agents for the treatment of neurodegenerative disorders and diseases.

Role of Cytokines in Central Nervous System Disease

An inflammatory response is one of the immune system s methods for dealing with microbial invaders, foreign proteins, and malignant cells. Effector cells of the inflammatory response migrate to the site of infection or injury where they are activated by cytokines, the ultimate regulators of this response. This response may be considered a necessary evil to combat foreign invaders and harmful malignancies, but also has the potential to cause damage to the involved tissues. Although most tissues in the body can tolerate inflammatory products and processes, the CNS is extremely sensitive to an inflammatory immune response. Thus, the CNS must maintain tight control of inflammatory responses and their mediators, the cytokines (13, 83, 151). Cytokines produced in the CNS influence both disease manifestations and infection outcomes (99).

Neurons and glial cells, in addition to being capable of responding to cytokines, are also able to produce and secrete many cytokines, including IL-1, IL-6, IL-10, TNF-α, TGF-ß, and IFN-α, -ß, and -γ (25, 46, 52, 81, 146). This ability of CNS cells to secrete and respond to cytokines allows these molecules to facilitate communication between the CNS and the immune system, as well as, communication within the CNS via autocrine or paracrine mechanisms (128). Unfortunately, these communications often have a

detrimental effect in the sensitive environment of the CNS, and cytokines have been implicated as pathogenic mediators in the CNS due to their various effects, such as the induction of fever or anorexia (7, 42, 117). The primary central effect, fever, results from the interaction of IL-1, IL-6, or TNF-α with the thermoregulatory neurons of the hypothalamus (42). Anorexia, part of the acute phase reaction to acute and chronic inflammatory and infectious diseases, is induced by IL-1 or TNF-α (15, 42, 107) via a prostaglandin-dependent mechanism which affects the activity of glucose-responsive neurons in the hypothalamus (42, 78).

In addition to their involvement in these pathogenic processes, cytokines have also been implicated in a number of neurodegenerative diseases including ischemia (stroke), multiple sclerosis, Alzheimer s Disease, cerebral malaria, bacterial meningitis, and Human Immunodeficiency Virus (HIV)-induced dementia (19, 50, 95, 142, 151, 156). In addition, it has been demonstrated that IL-1 and IL-6 levels in the CNS are increased immediately following a stabbing injury to the brain (151). Table 1 shows a partial list of CNS disorders that have been associated with the pathologies of proinflammatory cytokines. That these conditions represent a wide variety of different types of neurodegenerative diseases and disorders including injury, autoimmune disorders, and bacterial, viral, and parasitic infectious diseases, further illustrates the wide range of effects that cytokines can exert in response to a myriad of insults.

To elucidate further the role of cytokines in CNS pathologies, researchers have utilized transgenic mice with targeted expression of specific cytokines in their astrocytes. These mice exhib neurological disorders that can be correlated with the level of cytokine expression. Specifically, IL-6-expressing transgenic mice displayed decreased cognitive

Table 1. Partial list of human CNS disorders in which proinflammatory cytokines play a role in neurodegeneration and pathogenesis.

Disease/Disorder	Cytokines Implicated	Reference
Infectious Disease		
Bacterial Meningitis	IL-1, IL-6, IL-8, TNF-α	(119)
	anti-TNF and anti-IL-1 protective	
Cerebral Malaria	TNF-α	(53)
AIDS encephalopathy	TNF-α, IL-1, IL-6	(49)
VEE Virus	TNF-α, IL-6, IFN, IL-1	(124)
JE Virus	IL-8	(132)
Transmissible Subacute Spongiform Encephalopathy	IL-1, TNF-α	(21)
Autoimmune Disease		
Multiple Sclerosis	IFN-γ, TNF-α, IL-6, IL-1	(84, 151)
EAE	IL-1ß, IL-6, TNF-α, IFN-γ	(96)
Neurodegenerative Disorders		
Alzheimer s Disease	IL-1, IL-6	(38, 145)
Parkinson s Disease	IL-1, IL-6, TNF-α	(89, 90)
Injury		
Stroke/Ischemia	TNF-α	(37, 43, 44)
Stab wounds	IL-1, IL-6	(151)
Neuropsychiatric Disorders		
Adult schizophrenia	IL-1ß, IL-2	(73)
Obsessive-Compulsive Disorder	IFN-γ, IL-2, IL-12	(87)

ability and neurodegeneration (20), while TNF-α-expressing transgenic mice exhibit lymphocytic meningoencephalomyelitis with paralysis. These results combined with other studies have led these investigators to conclude that overexpression of particular cytokines, in an otherwise normal CNS, is pathogenic, and therefore, support the hypothesis that cytokines are involved in structural and functional changes observed in the CNS in human inflammatory neurological disorders (19, 20).

The mechanisms by which proinflammatory cytokines cause neurodegeneration vary. In Alzheimer's Disease it is probable that elevated IL-1 and IL-6 levels somehow stimulate the production of ß-amyloid protein precursor, which in turn produces ß-amyloid protein, known to activate apoptosis of neurons (38, 82). The mechanism by which cytokines are involved in HIV encephalopathy involves a positive feedback loop between macrophages and astrocytes. TNF-alpha and IL-1ß both increase arachidonic acid (AA) metabolism; the resulting AA metabolites then enhance TNF-alpha and IL-1ß production by macrophages, creating an amplification event in the CNS (51). In addition to enhancing proinflammatory cytokine production, these metabolites are also neurotoxic. It has been shown *in vitro* that HIV-1-infected monocytes produce large amounts of AA metabolites only in the presence of astrocytes. This finding suggests that astrocyte production of proinflammatory cytokines may contribute to neurodegeneration in HIV infection when associated with macrophages (51). As the majority of HIV-infected cells in the CNS are of macrophage lineage (microglia and infiltrating macrophages) (56), this model is certainly plausible. Although CNS infection occurs in essentially all of HIV-infected patients, only about 30% develop HIV dementia. In these cases, it is not the

viral titer that predisposes HIV patients to this outcome, but the quantity of proinflammatory cytokines secreted by activated macrophages in the brain (56).

In ischemia, the cytokine TNF-α plays a different role. One of the central components of ischemia is the rapid and persistent accumulation of polymorphic neutrophils (PMN) to the ischemic site. High levels of TNF-α co-localize at the site almost immediately after the ischemic event, suggesting the possibility that TNF-α may be responsible for the initiation of the PMN response (7). One study showed that intracerebroventricular injection of TNF-α 24 hours prior to middle cerebral artery occlusion in rats intensifies tissue injury induced by the ischemic event (10). Indeed, in experimental animal models, monoclonal antibodies to TNF-alpha reverse this effect (7). It has been suggested that TNF-α may activate the capillary endolthelium to a pro-adhesive state, thereby priming the brain for subsequent ischemic damage (37).

TNF-α, together with IFN-γ, IL-1, and IL-6, also seems to be involved in neurodegeneration associated with Multiple Sclerosis. This observation has been hypothesized to occur via an inflammatory cascade involving these cytokines which promotes demyelination and oligodendrocyte death (156).

In several diseases, the mechanisms by which cytokines contribute to neurodegeneration are unclear. Patients with pediatric obsessive-compulsive disorder exhibit elevated circulating levels of IFNγ L-2, and IL-12, while adult schizophrenic patients display increased circulating levels of IL-1ß and IL-2 . Exactly how these cytokines may be involved in the pathology of these neuropsychiatric disorders has yet to be determined (73, 87). In regards to inflammation as a result of Japanese encephalitis virus (JEV), a microbial infection, the mechanism by which the cytokine IL-8 is involved

is also unknown. In patients infected with JEV, a significant correlation exists between increased IL-8 levels and severity of illness (132). In all of these examples, proinflammatory cytokines have been associated in the pathogenesis of CNS diseases and disorders. However, it is also important to analyze the role of anti-inflammatory cytokines in ameliorating symptoms of particular CNS disorders. Furthermore, several CNS pathologies are marked by the reduced level or complete absence of anti-inflammatory cytokines as compared to healthy subjects.

Multiple Sclerosis (MS), a demyelinating disease of the CNS, is not only characterized by an increase in proinflammatory cytokines, but also by a decrease in anti-inflammatory cytokines including TGF-ß (58, 91). Experimental Autoimmune Encephalomyelitis (EAE) in rats and mice shares many similarities with MS and is used as an experimental model for the demyelinating disease. Administration of anti-inflammatory cytokines TGF-ß, IL-4, IL-10, and IL-13 have been shown to successfully ameliorate the symptoms of EAE (29). Currently, the only two drugs approved for the treatment of MS appear to be partially effective and work through their capacity to increase levels of TGF-ß, IL-4, and IL-10 (84).

Experimental autoimmune neuritis (EAN) in rats is the experimental model for Guillain Barr Syndrome, an acute demyelinating inflammatory polyneuropathy. EAN is also suppressed by treatment with TGF-ß (29).

The absence of anti-inflammatory cytokines, specifically IL-4 and IL-10, has been implicated in HIV dementia. HIV-positive patients not displaying dementia and HIV-negative individuals have IL-4 and IL-10 mRNA easily detectable in the brain (56, 148). In contrast, patients displaying HIV dementia have no detectable levels of IL-4 mRNA in

their brains (56) and IL-10 mRNA levels are decreased (148). It was hypothesized that in the absence of the cytokine IL-4, which downregulates macrophage activation (56), macrophages are activated uncontrollably, leading to neurotoxicity and dementia via the production of potentially toxic products (56). Thus cytokines, both proinflammatory and anti-inflammatory, are likely to be important components of CNS pathologies, as demonstrated by their varied involvement in a wide variety of CNS disorders.

Role of Neurotrophic Factors in Central Nervous System Disease

During neuronal development, if neuronal cells are deprived of neurotrophins such as NGF or BDNF, neuronal cell death will occur (4, 61, 69, 97). The general consensus is that neurotrophins play a protective role in these neuronal populations by suppressing the expression of genes involved in apoptosis (4, 69, 97). If levels of neurotrophic factors decline, then the loss of the transcriptional suppression in programmed cell death may occur, possibly resulting in neuronal decay as seen in normal aging or in the neuronal loss observed in neurodegenerative disorders, including Alzheimer s Disease (69). Current research is being conducted to elucidate the role of neurotrophins in neurodegenerative diseases such as Alzheimer's, Parkinson's, and Huntington's Diseases, as well as Amylotrophic Lateral Sclerosis (ALS or Lou Gehrig's disease). Such studies could lead to the possibility of therapeutic roles for neurotrophic factors in the treatment of these and other CNS diseases (27).

Much research has been conducted on the neurotrophic factor, nerve growth factor (NGF), which is considered to be the prototype neurotrophin (60). Decreased levels of NGF in the CNS are observed in several diseases, including alcoholic and diabetic

neuropathy and Alzheimer's disease (60). In one recent study, transgenic animals homozygous for NGF disruption resulted in considerable neuronal loss in both sensory and sympathetic ganglia (30). Other studies have shown that NGF can have a beneficial effect in aged rats with memory deficits (45, 68). NGF is of particular interest in the treatment of Alzheimer's Disease, specifically in reducing and/or preventing the cholinergic neuronal atrophy observed in Alzheimer's patients (116). When NGF was infused intracranially into an Alzheimer's patient, there was an increase in several indicators of improved cognitive performance, including verbal episodic memory (129). However, due to problems in administering NGF and its possible negative side-effects, more research needs to be conducted to understand the effects, both beneficial and detrimental, of NGF in the brain (27).

Another neurotrophin, BDNF, also has potential therapeutic applications in the treatment of Alzheimer s Disease. BDNF is widely distributed within the CNS and lends trophic support to several different populations of neurons, including cholinergic, dopaminergic, and serotinergic neurons and motor neurons (27). Of particular note is that BDNF messanger RNA (mRNA) expression levels are decreased in the hippocampus and temporal cortex of Alzheimer's patients as compared to normal controls (28, 94, 106). This decrease in BDNF mRNA and subsequent loss of BDNF s protective effects may contribute to the progressive neuronal atrophy associated with Alzheimer's Disease (27). Additionally, since BDNF plays a physiological role in learning and the formation of memory (39, 72), a loss of BDNF could worsen the cognitive deterioration seen in Alzheimer s patients (27). The capacity of BDNF to provide neuroprotection for

dopanergic and motor neurons has led to studies that examine the use of BDNF in the treatment of Parkinson's disease and other motor neuron diseases (27).

Another neurotrophin, Glial cell-line derived neurotrophic factor (GDNF), has also been investigated to determine its role in neurodegenerative disorders. Researchers have concluded that GDNF reduces the rate of apoptosis in cultured dopanergic neurons and prolongs neuronal survival (26). Studies have shown that GDNF's neuroprotective role in several neuronal populations have made this neurotrophin desirable as a therapeutic candidate in several neurodegenerative diseases, including Alzheimer's Disease, Parkinson's Disease, Huntington's Disease, and ALS (5, 16, 27, 102, 120, 135). Table 2 is a partial list of neurotrophins whose dysregulation has been implicated in neurodegenerative disorders and the disorders in which they are being investigated as therapeutic treatments.

In addition to the progressive neurodegenerative diseases listed above, neurotrophins are protective in the CNS against a variety of insults or injuries. Recent studies have shown that the production of the proinflammatory cytokines IL-1ß and TNF-α activate production of neurotrophins in the CNS, thus implicating neurotrophins in the early processes of neuronal regeneration in response to microbial invaders and injury (59). Consequently, neurotrophins appear to play an important protective role in the CNS.

Introduction to Venezuelan Equine Encephalitis Virus

Background and Discovery

Discovered in 1939, Venezuelan equine encephalitis virus (VEE), an alphavirus of the *Togaviridae* family, causes mosquito-transmitted encephalitic endemics in North and

Table 2. Neurotrophins in disease.

Neurotrophin	Dysregulation in Disease	Main Therapeutic Benefit	Other Therapeutic Benefits	Reference
BDNF	↓ AD	AD	PD ALS	(27, 28, 94, 106)
CNTF	↓ PD ↓ AD	ALS	AD PD	(64, 118)
GDNF		PD	AD HD ALS	(5, 16, 27)
NGF-ß	↓ AD ↑ HSV ↓ Neuropathies	AD Peripheral Neuropathies	PD	(60, 116)

AD - Alzheimer s Disease
HD - Huntington s Disease
PD - Parkinson s Disease
ALS - Amylotrophic lateral sclerosis
HSV - Herpes simplex virus

South America (18, 77). Although primarily found in Venezuela, this pathogen has caused outbreaks as far north as south Texas and Florida (155). VEE is endemic to horses and rodents but is capable of causing disease in humans. Epidemics of VEE occur approximately every two to ten years, the most recent occurring in Columbia in 1995, where over 12,000 people were infected (88). Symptoms of VEE in humans are often incapacitating and include fever, severe headache, muscle pains, and a skin rash. Encephalitis develops in less than 0.5% of infected adults and in approximately 4% of infected children (104). Mortality is less than 1% of those infected, but in children, especially those in areas with poor medical support, it may be as high as 4% (104). The only available treatment is supportive therapy.

Properties of the VEE virion

VEE virion structure and pattern of gene expression are typical of the alphavirus genus, of which Sindbis virus is the prototypical example (34, 104). The non-segmented single-stranded positive-sense RNA genome is encapsidated in an icosahedral nucleocapsid, which is composed of multiple copies of a single polypeptide, the C protein (35, 137). Two viral glycoproteins, E1 and E2, form heterodimeric spikes on the host cell-derived viral envelope (101, 122). These glycoprotein spikes are necessary for viral attachment and penetration, as well as interaction with the host immune system (33, 34, 112). The genome of the VEE is 11,444 nucleotides long (76), shares all features of Sindbis virus (e.g. capped, polyadenylated, active 26S subgenomic mRNA promoter, two segregated regions coding for non-structural and structural proteins respectively) and can be infectious under appropriate transfection conditions (34). VEE research has been

significantly advanced since the molecular cloning of the VEE genome (36). The resulting infectious full-length complementary DNA (cDNA) clone, in which the VEE sequence was placed downstream of a bacteriophage T7 promoter in an expression system, has been modified using site-directed mutagenesis to produce a panel of attenuated cloned VEE mutants. As shown in Figure 3, these mutant VEE clones differ from their virulent parent in specific bases that result in single amino acid substitutions (35, 55).

Despite the fact that the parent and all mutant viruses replicate to similar titers in the brains of peripherally injected mice (with the exception of the non-neuroinvasive V3032), mortality rates vary (35, 55, 124). These mutants were selected for their accelerated penetration, a trait which coincided with attenuation (70). A possible explanation for the different mortality rates may be a common evolutionary event. Virus size precipitates a need for economy: viral genomes must be efficient and not carry unnecessary genes. Therefore, it is not uncommon for viruses to enhance one trait at the expense of another. In the case of accelerated penetration in VEE, it may be that these mutants become so specialized at penetration, that another trait, their ability to be pathogenic, is diminished (70).

Aternatively, possible explanations for this phenomenon may come from studying parallels in other viruses, such as HIV. Research has indicated that neurotoxicity as a result of HIV-1 infection may be primarily mediated by an HIV envelope glycoprotein, gp120 (127). HIV-1 binds to microglia and macrophages in the brain via an interaction between gp120 and the CD4 receptor and initiates a cell signaling cascade that induces the release of several potentially neurotoxic cytokines, including IL-1 and TNF-α

Table 3. Genotypes and phenotypes of selected molecularly cloned VEE strains.

Virus	**Envelope Glycoprotein Spike Amino Acid (AA) Position and AA**			*Phenotype*	*Mortality Rate*
	E2	E2	E1		
V3000	WT	WT	WT	Neuroinvasive, neurovirulent	100%
V3010	***E76K***	WT	WT	Neuroinvasive, neuroattenuated	10%
V3032	WT	***E209K***	WT	Non-neuroinvasive, Neurovirulent	0%
V3034	WT	WT	***A272T***	Neuroinvasive, neuroattenuated	20%

Amino acids listed in ***italics*** are the only changes between a given attenuated VEE mutant and the VEE parent, V3000. WT = wild type. Mortality rate is in adult C57BL/6J mice infected via left rear footpad injection. Modified from (55), with permission of Dr. Franziska Grieder.

(49, 85, 86, 141). Additionally, gp120 induces apoptosis in neurons via a mechanism where an increase in internal calcium ion concentration triggers a cascade of molecular events, including activation of third messengers that activate apoptosis or inactivate apoptosis inhibitors (1, 8, 127). If HIV-1 neurotoxicity is mediated by an envelope glycoprotein, it is also possible that VEE neurotoxicity is also mediated by an envelope glycoprotein. Hence, mutating the envelope glycoproteins of VEE could attenuate the strain and illicit an altered cytokine profile, despite the ability of the different strains to replicate to similar titers in the brain. Whatever the underlying mechanism of attenuation in the mutated strains, they provide a useful tool for studying VEE pathogenesis.

Pathogenesis of VEE

After mosquito inoculation, VEE spreads through the locally draining lymph nodes, establishes viremia, and disseminates to other lymphatic organs (55). This first phase of VEE spread is followed by a second, neuronal, phase (6). Although VEE entry into the CNS has been demonstrated to occur via the olfactory neuroepithelium, evidence also exists that viral entry may occur through capillary endothelial cells, and the exact entry mechanism into the CNS is, as yet, unknown (24). Once in the CNS, VEE spreads easily, infecting both neurons and glial cells (125). In addition to neuronal cell death, VEE causes gliosis and an intense inflammatory response (55, 66). VEE appears to have a direct association with neurodegeneration, as VEE antigens can be localized in a subset of dead neurons (67). However, in a different subset of dead neurons, no VEE antigen could be found, suggesting an alternate, indirect mechanism of neuronal degeneration.

As glial cells, such as microglia and astrocytes, have been shown to be infected via the presence of viral antigen (67, 124, 125), one possible explanation for this phenomenon is that neuronal death occurs following loss of support from glial cells.

Microglia and astrocytes play a critical role in the support and protection of neurons. Astrocytes, the most predominant glial cell in the CNS, outnumber neurons eight to one and are responsible for presenting antigen, secreting cytokines, phagocytosing debris, the buffering of ions, and the synthesis of essential neurotrophic factors, including BDNF, GDNF, NGF, and CNTF (11, 22, 75, 81, 138). Microglia play a role in CNS immune functions by acting as the resident macrophages of the brain, secreting cytokines and growth factors, and producing reactive oxygen and nitrogen intermediates (9, 11, 22, 138). Obviously, given these glial cells importance in neuronal support and protection, disruption of such support by VEE infection could contribute to VEE pathogenesis in the CNS.

Role as an Agent of Biowarfare

VEE also poses a threat as a biological warfare agent. VEE, under the military code name NU, was weaponized by the United States Army in the 1950 s and 1960 s (109, 143) and stockpiled at the Army's Pine Bluff Arsenal in Arkansas until 1972. It was destroyed in accordance with President Nixon's 1969 ban on the production of chemical and biological weapons (109). In addition, other countries, including the former Soviet Union, were known or suspected to have weaponized this virus as well. It can be produced in either a wet or dry form, is easily stabilized for weaponization, and can be delivered as an aerosol cloud or disseminated from a missile warhead (143). VEE is still

included on the official list of chemical-biological-radiation warfare agents and is, therefore, considered a serious public health threat.

Inflammatory Response to VEE

As has been discussed previously, a powerful immune response to a microbial invader such as VEE is necessary for clearance of the organism and the ultimate survival of the host. However, as has also been discussed, a too vigorous or imbalanced inflammatory immune response in the CNS can be detrimental. This process would appear to be the case in the pathogenesis of VEE. Studies involving immunodeficient mouse strains provide strong evidence that a robust immune response plays a crucial role in the development of viral encephalitis. A key observation in support of this notion is that severe combined immunodeficient (SCID) mice infected with virulent VEE have increased survival compared to immunocompetent mice infected with the same virus (23). Further, SCID mice develop a persistent, nonfatal infection following exposure to another alphavirus, neurovirulent Sindbis virus, that causes 100% mortality in immunocompetent mice (149). Collectively, these results suggest that a reduced inflammatory response may be beneficial for a positive outcome following VEE-induced encephalitis. My thesis has been to characterize the CNS response to VEE by examination of the production of cytokines and neurotrophins in the CNS following infection by both neurovirulent and neuroattenuated molecularly cloned strains of VEE. The ultimate goal of this research is to identify both beneficial and detrimental responses to VEE and exploit these responses for maximal potential therapeutic benefits.

MATERIALS AND METHODS

In Vivo Experiments

Animals

Six to 8-week-old female C57BL/6J (B6) mice (Jackson Laboratory, Bar Harbor, ME) were used for all experiments. Five mice per treatment group were utilized for these experiments; each experiment was reapeated three times. All mice were lightly anesthetized with methoxyflurane (Pitman Moore Inc., Washington Crossing, NJ) and infected with 1 x 10^3 PFU/25 µl PBS via left rear footpad injection (f.p.) with one of the VEE strains or mock-infected with PBS, as previously described (124). To confirm that surviving mice had acquired a VEE protective immune response, these mice were challenged with a lethal dose of virulent V3000 (1 x 10^4 PFU/100 l PBS) via intraperitoneal injection and observed for 14 days post-infection. The failure of mice to succumb to this dose of VEE was taken as evidence of an active immune response to initial exposure.

Virus strains

Three neuroinvasive, molecularly cloned VEE strains, virulent V3000, attenuated V3010 and V3034, were used for all experiments (35, 55). The virulent V3000 clone, with a mortality rate of 100% in C57BL/6J (B6) mice (36), was derived from a Trinidad donkey strain of VEE (108). The two neuroattenuated VEE mutant clones were created by site-directed mutagenesis of V3000 targeted at specific sites on one of the two envelope glycoproteins, E1 and E2 (35); these mutants differ from their parent by only

single amino acids (34, 35, 55). In V3010, a lysine is replaced by a glutamic acid in glycoprotein E2 at position 76, which results in a reduced mortality rate of 10% in B6 mice via footpad injection (35). Using site directed mutagenesis, an alanine was replaced with threonine in E1 at position 272 in the neuroattenuated V3034 mutant. The mortality rate of this mutant is 20% in B6 mice when injected by the same peripheral route (55). Molecularly cloned virus stocks were stored at —80˚ C, and all experiments were conducted in a biosafety level 3 laboratory.

Virus titration

Six to 8-week-old female B6 mice were infected with 1 x 10^3 plaque-forming-units (PFU) of V3000, V3010, or V3034 via left rear footpad injection (n = 10 animals per treatment group). For each treatment group, two mice were sacrificed at 3, 4, 5, 6, and 7 days post-infection, and brains were collected for titration of virus. Brain samples were homogenized in four volumes of phosphate buffered saline with Ca^{2+}/Mg^{2+} and 0.1% donor calf serum (PBS) solution, resulting in a 20% (weight/volume) suspension, and frozen prior to titration, as previously described (55, 124). Plaque assays on BHK-21 cells (American Type Culture Collection, Rockville, MD) between passages 53 to 63 were used to determine viral titers and confirm viral inocula concentrations (121, 125). Virus titers in the CNS were calculated as PFUs per gram of tissue with a detection level of 1.65 x 10^2 PFU/g tissue.

Extraction and detection of mRNA

B6 mice were infected with 1 x 10^3 PFU/25 ml PBS of virulent V3000,

neuroattenuated V3010 or V3034, or mock-infected with PBS via f.p. injection. Total cellular RNA was harvested on days 3, 4, 5, 6, and 7 post-infection from the hemi-sectioned cerebra of three mice for each treatment group. Samples were homogenized using a polytron (Kinematica AG, Switzerland) in a final volume of 1 ml of RNAzol™ B (Tel-Test, Inc., Friendswood, TX). After RNA extraction, the concentration and purity of RNA in each sample was determined spectrophotometrically (Beckman Instruments, Inc., Columbia, MD) by assessing the $A_{260/280}$ ratios, as previously described (124). Specimens were stored at —80˚C until processing by Ribonuclease Protection Assay (RPA) using the Riboquant® System (Pharmigen, San Diego, CA).

RPA s were carried out to detect specific mRNA species. Three multi-probe templates were used to screen for the upregulation of specific genes. These templates, rNT-1, mCK-2b, and mCK-3b, are listed in Table 4. ^{32}P-labeled anti-sense RNA probes were synthesized by *in vitro* transcription and hybridized in excess of 15 µ g of target RNA in hybridization buffer at 56^{o}C for 14-16 hours, after which free probe and other single-stranded RNA species were digested with Rnases, as previously described (124). The remaining protected RNA probes were extracted, purified, and resolved on a denaturing 6% polyacrylamide gel. Gels were blotted, dried under vacuum at 80^{o}C for 1 hour and exposed to Kodak X-OMAT™AR film (Sigma, St. Louis, MO) for various times ranging from 6 to 24 hours at —80˚C to obtain optimal exposure. Autoradiograms were scanned into a digital image and blots quantified by measuring pixel densities. Pixel densities were measured using ScionImage software for Windows (Scion Corp., Frederick, MD) and ratios of genes of interest to the constitutively expressed glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene were generated. Means (±

Table 4. RNase protection assay templates.

rNT-1	mCK-2b	mCK-3b
NGF-_	IL-12p35	TNF-ß
BDNF	IL-12p40	LTß
GDNF	IL-10	TNF-α
CNTF	IL-1α	IL-6
NT-3	IL-1ß	IFN-γ
NT-4	IL-1Ra	IFN-ß
L32	IL-18	TGFß1
GAPDH	MIF	TGFß2
	IL-6	TGFß3
	IFN-γ	MIF
	L32	L32
	GAPDH	GAPDH

Three templates were used to analyze brain or astrocyte samples for inducible genes which include a neurotrophic factor template (rNT-1) and two templates for cytokines (mCK-2b and mCK-3b). Each template includes two constitutively expressed genes, L32 and GAPDH, as internal controls. Modified from (124).

S.E.M.) were calculated from three independent samples and relative changes in gene expression were then based on comparisons to the mock-infected group. Assay controls included undigested ^{32}P-labeled probes, unprotected RNA treated with RNase, and RNA harvested from resting and LPS-stimulated mouse macrophages.

Statistical analysis

Data were analyzed using the software program SPSS 8.0 for Windows' or SigmaStat 2.0 (SPSS, Inc., Chicago, IL). Gene expression data was analyzed using one way analysis of variance (ANOVA) in order to determine whether groups differed based on treatment. Least significance difference (LSD) post-hoc tests or Tukey tests were then used to determine which treatment groups differed significantly from controls and whether differences were demonstrated among treatment groups. All analyses were two-tailed with statistical difference established at $p<0.05$ a priori.

Astrocyte experiments

Primary astrocyte cultures

Primary astrocyte cultures were established by removing the cerebra of 2-day-old Sprague-Dawley rats via sterile technique. Microscopic dissection was used to remove the meninges. Cerebral hemispheres were dissociated by suction pipetting followed by centrifugation at 1000 r.p.m. for 10 minutes. Supernatants were removed, and each cell pellet resuspended in cell culture media. Suspensions were then sequentially triturated through 18 and 22 gauge needles. The resulting cells were plated in Dulbecco s minimum essential media (DMEM) supplemented with 10% non-heat inactivated fetal

bovine serum, 1% L-glutamine, and 25 g/ml gentamycin. Adherent microglia were removed by rotary shaker after the establishment of a confluent monolayer, as previously described (125). Astrocytes were characterized by positive immunofluorescent staining for glial fibrillary acidic protein (GFAP; Sigma, St. Louis, MO), an astrocyte-specific marker. The homogeneity of these astrocytes cultures was ascertained via immunostaining identical cultures with a microglia-specific cell surface marker, OX42 (Serotec/Harlan, Indianapolis, IN), and an oligodendrocyte-specific marker, antiGal-C (Boehringer, Germany). The astrocyte primary cell cultures were determined to be greater than 95% homogenous by three independent samplings of GFAP-stained cell cultures and comparing these cell counts to phase contrast light microscopy, as previously described (125). Only on rare occasions were contaminating microglia and oligodendrocytes observed.

Virus growth kinetics in astrocytes

Viruses used to infect astrocyte cultures were the same as those described above for *in vivo* methods. Astrocytes were passaged one time and plated into 60 mm cell culture plates (Corning, NY, USA). Cultures were incubated for four days at 37^{o}C, 5% CO_2 until 90-95% confluent. Astrocyte cell cultures were infected in triplicate with virulent V3000 or the attenuated V3010 at a multiplicity of infection (MOI) of 1.0 and incubated for 1 hour at 37^{o} C, 5% CO_2. Astrocytes were then washed with PBS containing 0.1% donor calf serum, and 3 ml of media was replaced. Samples were collected at 2, 6, 12, 24, and 48 hours post-infection. Supernatant samples containing virus were immediately frozen

at —80°C. Virus titers were determined by plaque assays on BHK-21 cells as described above in mouse experimental protocols.

Extraction of astrocyte mRNA

Astrocytes were passaged one time and plated into 6-well culture plates and incubated at 37° C, 5% CO_2 for two days. Cell counts were determined to be 1 x 10^6 cells per well. Medium was removed and wells were assigned to one of four different groups: uninfected PBS control, recombinant rat IFN-γ (100 U/ml, Genzyme Corp., Cambridge, MA) and LPS (1 g/ml protein-free *E. coli* K235 LPS, kindly provided by Dr. Stefanie Vogel, USUHS, Bethesda, MD) stimulated, virulent V3000-infected or attenuated V3010-infected, both at MOIs of 1.0, and incubated for one hour. Total cellular RNA was collected from three different samples per treatment group at 2, 6, 12, 24, and 48 hours post-infection using RNAzol"B. RNA was extracted with chloroform (Sigma, St. Louis, MO), precipitated with isopropanol (Sigma, St. Louis, MO) and diluted in diethylepyrocarbonate (DEPC)-treated water (Quality Biologicals, Inc., Gaithersburg, MD) as previously described (125). The concentration and purity of RNA was determined as described above in mouse experimental protocols. Specimens were stored at —80°C until processed.

Detection of astrocyte mRNA

Expression of mRNA was detected by two methods. One method was the RPA, as described above, and the other method was via reverse transcription-polymerase chain reaction (RT-PCR). For the latter method, cDNA synthesis was performed by reverse

transcription using 200 U Moloney Murine Leukemia virus (MMLV) reverse transcriptase (GIBCO, Gaithersburg, MD) in a 25 l reaction volume following standard protocols. Amplification of cDNA was accomplished using gene-specific sense and anti-sense oligonucleotide primers for rat GAPDH, TNF-α, IL-1ß, IL-6, and TGF-ß. Products amplified from cDNA could be distinguished from genomic DNA because primers were designed to span at least one intron. The PCR reaction mixture containing 1 U Taq polymerase (Promega, Madison, WI) was amplified using an automated PCR thermocycler (Perkin-Elmer, Norwalk, CT). Ten l of amplified PCR products along with 2 l of gel loading buffer were added to each well in a 1.5% agarose gel and electrophoresed at 100 V for 45 to 50 minutes in 1X TBE buffer. Following electrophoresis, the gels were denatured, neutralized, and transferred to Hybond N^+ membranes (Amersham Life Science, Arlington Heights, IL) using 10X SSC by standard capillary Southern blotting techniques (134). DNA was then cross-linked to the membrane by exposure to UV light for two minutes and baked at 80^o C in a vacuum oven. Subsequent visualization of specific DNA bands on the blots was conducted using fluorescein labeled-oligonucleotide probes complimentary to the PCR products and detected using the enhanced chemical luminescence technique (ECL™, Amersham LifeScience, Buckinghamshire, England), that was then scanned into a digital image and quantified as described above.

Cytokine immunoassays

TNF-α, IL-6, and IL-1ß proteins in primary astrocyte culture supernatant were measured using the Quantikine® M rat TNF-α, IL-6, or IL-1ß kit, respectively (R&D

Systems, Minneapolis, MN) as per standard manufacturer Enzyme-Linked-Immuno Sorbent Assay (ELISA) protocols. Supernatants were harvested from three independent wells for each treatment group at 2, 6, 12, 24, and 48 hours post-infection and media replaced after each sampling. Samples were stored at 4^{o} C and were diluted 1:2 with the calibrator diluent before assaying. This step ensured cytokine levels were within the range of standards. Standards, controls, and samples were assayed in duplicate at a wavelength of 450 nm and 550 nm for wavelength correction. Optical density (O.D.) was determined as the change in O.D. between the two wavelengths and was quantitated on a microplate reader (Elx800, Biotek Instruments, Inc., Winooski, VT). A range of cytokine dilutions was used to generate a standard curve to determine the cytokine concentrations in the sample supernatant.

Statistical analysis

Data from the astrocyte experiments were analyzed as described above in mouse experimental protocols.

RESULTS

CYTOKINE AND NEUROTROPHIN RESPONSES IN THE CNS FOLLOWING VEE INFECTION

Proinflammatory Cytokine Responses

Interleukin-1ß

IL-1ß is a potent inflammatory cytokine. Neurons and astrocytes are both capable of producing and responding to this cytokine, but the main cellular source of IL-1ß in the brain appears to be the microglia (128). IL-1ß is expressed rapidly in the CNS following injury and/or microbial invasion and, in turn, initiates a cytokine cascade. Specifically, it has been demonstrated that IL-1ß is capable of stimulating the production of a number of both pro- and anti-inflammatory cytokines, including TNF-α, TGF-ß, and IL-8 (128).

IL-1ß mRNA expression is increased in the CNS following many types of injury to the brain (7, 62, 124). In ischemia, IL-1ß mRNA expression increases immediately, peaks at approximately 12 hours post-insult, and returns to basal levels by 5 days post-ischemia (7). IL-1ß is further implicated in the neuropathogenesis of ischemia with the finding that IL-1 receptor antagonist (IL-1ra) blocks the action of IL-1ß by binding available receptors and appears to be protective when administered following an ischemic event (113). In addition to ischemia, IL-1ß has been implicated in the neuropathology of certain viruses. Human T-lymphotrophic virus (HTLV)-1 associated myelopathy/ Tropical spastic paraparesis (HAM/TSP) involves the degeneration of the spinal cord associated with demyelination (65). In adult T cell leukemia patients seropositive for

HTLV-1 or asymptomatic carriers, IL-1ß levels are not elevated, while in contrast, IL-1ß levels are significantly elevated in patients with HAM (140).

IL-1ß is also implicated in the CNS inflammatory response to alphaviruses. Another member of the alphavirus family, Sindbis virus (SV), replicates in a manner similar to VEE, causing viremia before entering the CNS (17). Indeed, SV is a good model to investigate the pathogenesis, neuroinvasiveness, and neurocytotoxic mechanisms of alphaviruses (17, 40, 80). One experimental, attenuated strain of SV, TE12, is non-fatal in mice, and causes an acute, usually asymptomatic encephalomyelitis. A neuroadapted strain of SV, NSV, causes encephalitis with 100% mortality in mice, despite the fact that viral replication in the brain is similar to that of the more neuroattenuated TE12 strain. Of importance is the observation that in BALB/cJ mice, the inflammatory response (including IL-1ß expression) to the neurovirulent NSV is increased when compared to the response to neuroattenuated TE12 (149). Interestingly, immunodeficient SCID mice infected with the neurovirulent NSV strain displayed no mortality, although the virus replicated to similar titers as observed in BALB/cJ mice (149). These findings suggest that the CNS inflammatory response plays a role in SV neuropathology since the reduction of or absence of an inflammatory response in the immunodeficient host contributes to an avirulent outcome (149).

The induction of IL-1ß mRNA in the CNS following SV infection is rapid and occurs within 24 hours post-infection (114, 149). This rapid induction of IL-1ß mRNA peaked at day-four post-infection and slowly returned to near basal levels by day-20 (149). However, IL-1ß mRNA expression in the CNS of immunodeficient SCID mice

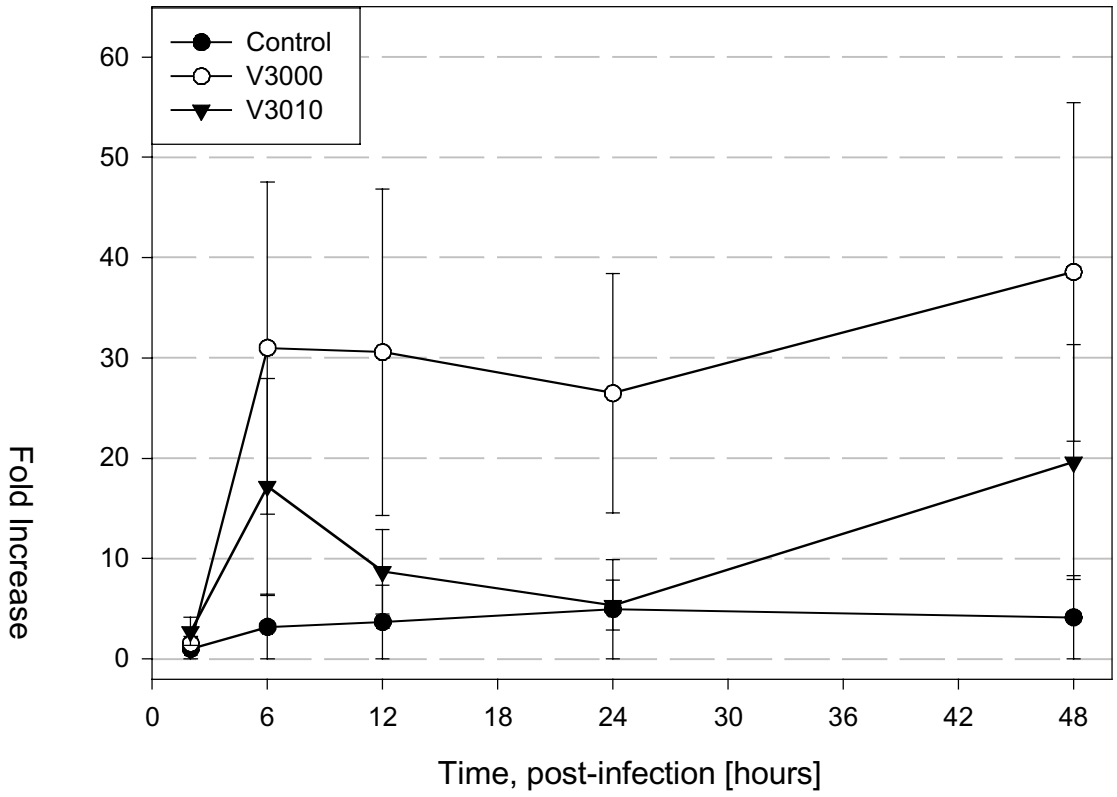
infected with the neurovirulent NSV was shown to be significantly lower, peaked at day-two, and returned to basal levels by day-10 post-infection (149).

Semliki Forest virus (SFV), another member of the alphavirus family, causes a non-fatal CNS infection with viral clearance by day-10 post-infection in mice infected with an avirulent strain (92). Of note is the observation that in this non-fatal alphavirus-induced encephalomyelitis, IL-1ß secretion in the CNS is only marginally increased as compared to normal controls in Biozzi ABH mice (92), supporting the hypothesis that proinflammatory cytokine induction in the CNS following alphavirus infection may contribute to neuropathogenesis and disease development.

My experiments with VEE have shown similar results. Primary astrocyte cultures infected with the virulent V3000 strain of VEE displayed a trend of increased upregulation of IL-1ß mRNA over the 48 hour time course as compared to similar primary astrocyte cultures infected with the attenuated V3010 strain, although the increased upregulation was not significantly different between the two primary astrocyte cultures infected with the different viruses (Figure 1). Although IL-1ß mRNA levels are increased in response to both viruses in primary astrocyte cultures as compared to mock-infected controls, these differences did not appear to be statistically significant.

I next investigated IL-1ß production by primary astrocyte cultures infected with either the virulent V3000 strain of VEE or the attenuated V3010 strain. At early time points, astrocytes infected with the virulent strain produced significantly higher amounts of IL-1ß than did astrocytes infected with the attenuated V3010. This V3000 upregulation of IL-1ß was seen as early as two hours post-infection, peaked at 12 hours

Figure 1. IL-1ß gene expression in astrocytes.

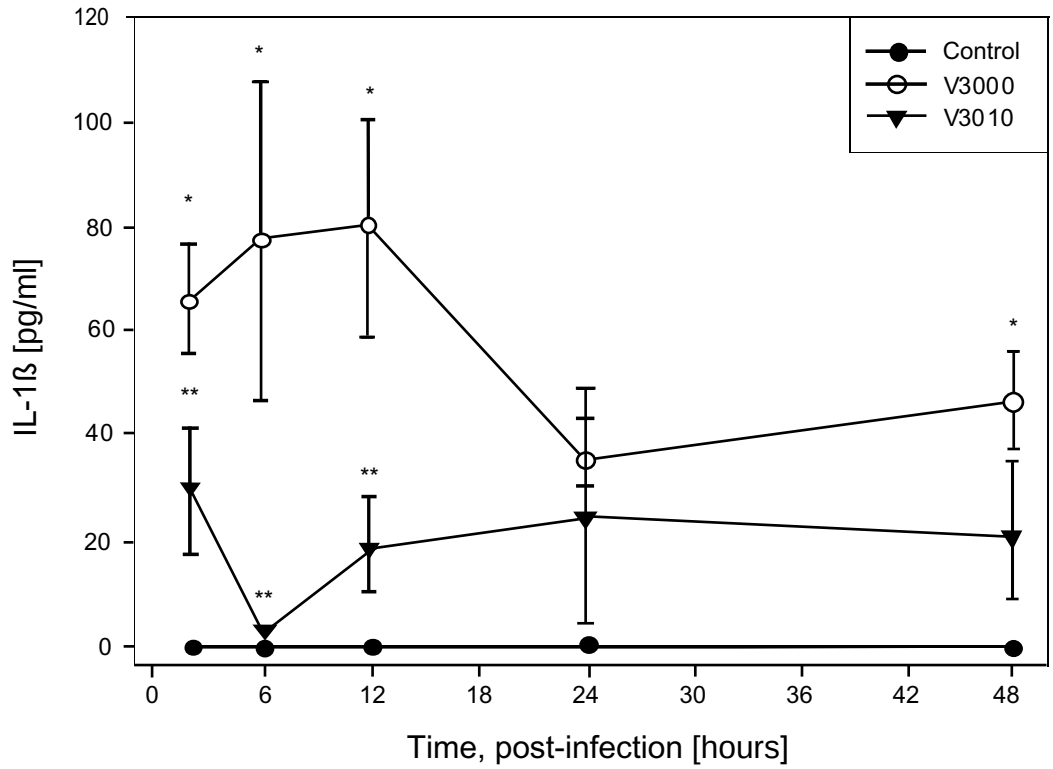


Primary rat astrocyte cultures were infected with virulent V3000 or attenuated V3010 at an MOI of 1.0. Total cellular RNA from triplicate samples was collected at 2, 6, 12, 24, and 48 hrs p.i. cDNA was synthesized and amplified via RT-PCR. Amplification products were subjected to gel electrophoresis, transferred to membrane via Southern blot, and visualized via ECL. Autoradiograms were scanned to a digital image. Gene expression was quantitated by measuring pixel densities of autoradiograms from three independent assays, determining ratios of inducible genes over the constitutively expressed GAPDH gene, and expressed as a relative fold increase (mean ± S.E.M.) as compared to mock-infected controls. There was no statistical difference between treatment groups.

post-infection, and remained elevated throughout the 48 hour time course. In contrast, astrocytes infected with the attenuated V3010 displayed significantly lower upregulation of the pro-inflammatory cytokine throughout the early period of the 48 hour time course, as seen in Figure 2. The significantly higher amounts of IL-1ß protein production seen in astrocytes in response to the two different VEE strains without a statistically different amount of mRNA present could possibly be due to different post-transcriptional or post-translational modifications occurring following infection with the different viral strains.

The results of our *in vivo* experiments in mice further demonstrate a difference in the relative capacity of virulent versus avirulent strains to elicit IL-1ß gene expression *in vivo* (116). Adult B6 mice displayed early upregulation of several genes encoding pro-inflammatory cytokines. This induction continued throughout the seven day experimental time course (124). However, strain, age- and sex-matched mice infected with the attenuated V3010 strain failed to induce a detectable pro-inflammatory cytokine gene response in the CNS at any point during the seven day time course (124). Furthermore, the attenuated V3034 infected mice displayed a delayed pro-inflammatory cytokine gene response, in that the upregulation was not seen until day five following infection (124). The different quantitative cytokine induction following *in vivo* infection with the three VEE strains occurred even though the kinetics and titers of virus in the CNS remain similar throughout the seven day time course (124). Although the precise mechanism responsible for this observation is unknown, it is possible that the various VEE strains induce different cell signaling cascades that in turn affect which cytokines are produced.

Figure 2. IL- 1 ß protein production in astrocytes.



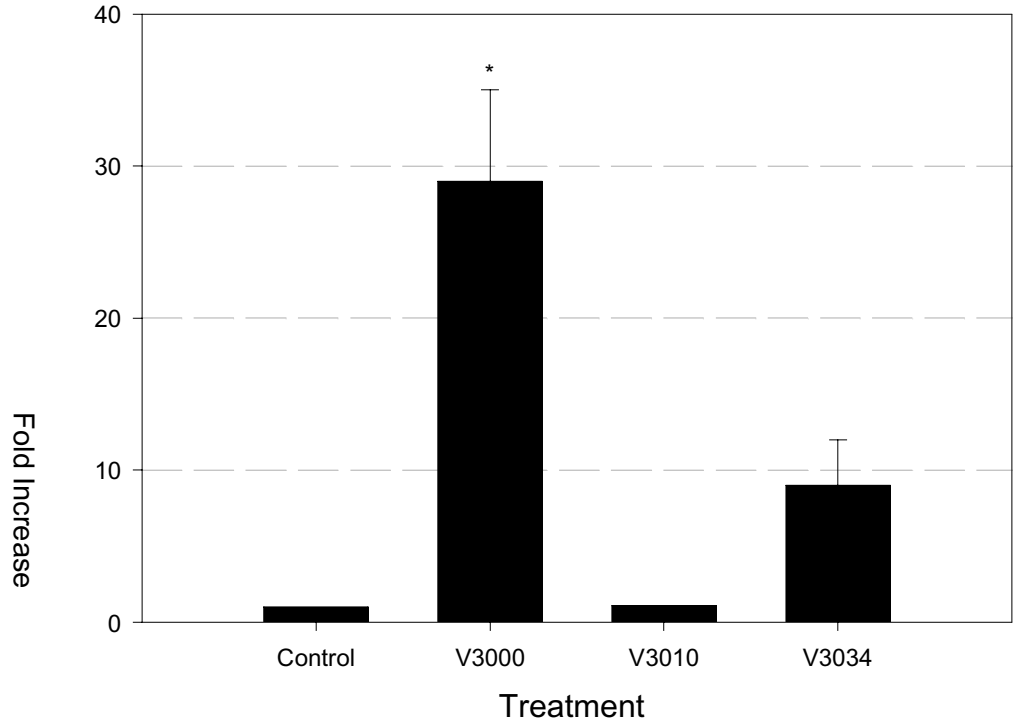
Astrocyte supernatants were analyzed for IL-1 ß production via ELIS A following infection with either the virulent V3000 or the attenuated V3010 strain. V3000 infection induced astrocytes to produce IL-l ß at significantly higher levels ($*p<0.05$) at all time points except 24 hrs. Attenuated V3010 infection of astrocytes resulted in significantly lower levels of IL-l ß as compared to virulent V3000 at 2, 6, and 12 hrs p.i. (** $p<0.05$)

In our studies, IL-1ß is the pro-inflammatory cytokine gene that showed the most remarkable increase in comparison to control mice; 30-times the quantity seen in control mice were observed (124). These findings are shown in Figure 3. The virulent V3000 strain significantly induced IL-1ß mRNA expression in the CNS as compared to either attenuated strain V3010 or V3034, or to controls, possibly due to altered cell signaling (124). Taken together, these studies suggest that the significant upregulation of this potent pro-inflammatory cytokine contributes to the significantly higher mortality observed in mice infected with the virulent strain.

Interleukin-6

When stimulated by IL-1 or TNF-α, both astrocytes and microglia are capable of producing IL-6 (46, 81, 128). Although described here as a proinflammatory cytokine, IL-6 displays both proinflammatory and anti-inflammatory properties (2, 7). IL-6 is consistently produced following many types of CNS injury including ischemia and blunt trauma (7). However, a proinflammatory role, rather than an anti-inflammatory role, is suggested by the finding that transgenic mice overexpressing IL-6 in the CNS display severe neuronal pathologies and several components of neurodegeneration, including astrogliosis, cerebral edema, and disruption of the blood brain barrier (20). IL-6 influences immune responses and inflammatory reactions and is a major physiological mediator of the acute phase response (64). Since IL-6 overexpression has been implicated in neurodegeneration (20), it is not surprising that the presence of this cytokine in the CNS has been implicated in neuropathogenesis of the CNS disorder

Figure 3. IL-1ß gene expression in mice.



Six to 8-week-old female C57BL/6J mice were infected with 1 x 10^3 PFU of attenuated V3010 or V3034 or virulent V3000 via left rear footpad injection. Brains were harvested daily on days 3 - 7 p.i. Gene expression was quantitated as a total for the entire experimental time course by measuring pixel densities of autoradiograms from three independent assays (n = 15 mice per group) and determing ratios of inducible genes over the constitutively expressed GAPDH gene and expressed as a relative fold increase (mean ± S.E.M.) as compared to mock-infected controls. IL-1ß genes were significantly upregulated following infection with virulent V3000 (*p<0.05). Modified from (116), used with permission of Dr. Bruce Schoneboom.

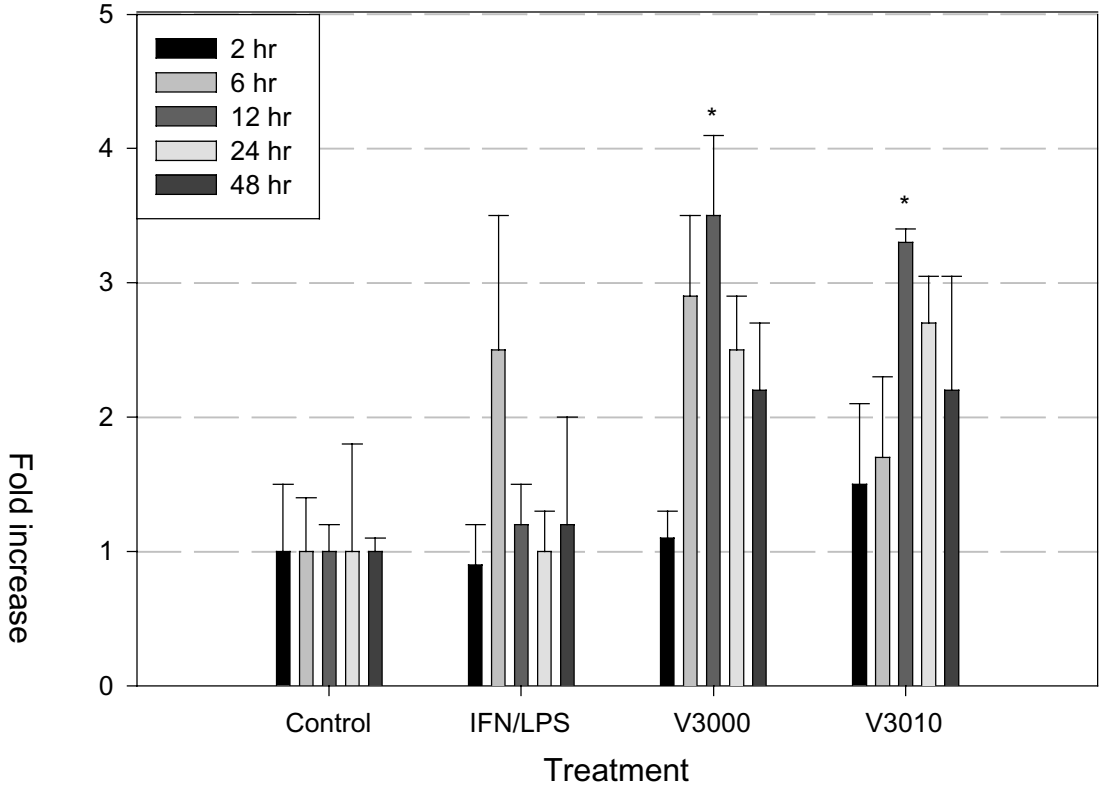
Guillain Barr Syndrome (GBS). GBS patients display elevated levels of IL-6 in their cerebrospinal fluid, but the significance of this finding is not yet known (128).

Like IL-1ß, IL-6 mRNA expression is increased in the CNS following alphavirus infection. Following infection with SV, several mouse strains show upregulation of IL-6 mRNA in the CNS very similar to that of IL-1ß (92, 149). Like IL-1ß, this upregulation occurred rapidly, within 24 hours after infection, and peaked by day four. However, unlike IL-1ß mRNA expression in the CNS following infection with the same virus, IL-6 mRNA expression rapidly fell after its peak and returned to basal levels by day 14 post-infection in BALB/cJ mice (149). In contrast to BALB/cJ mice, SCID mice infected with the neurovirulent NSV strain showed significantly decreased levels of IL-6 mRNA in the CNS (149). Similarly, in an *in vitro* study, microglia and astrocyte cultures infected with a different neurovirulent strain of SV significantly increased production of IL-6 as compared to identical cultures infected with a non-neurovirulent strain of SV (17).

In contrast to the kinetics of IL-6 mRNA expression following SV infection, non-fatal SFV infection in Biozzi ABH mice does not induce IL-6 secretion in the CNS until day 10 post-infection. This IL-6 secretion stays elevated until day 28 post-infection when it finally decreases, returning to basal levels by day 35 post-infection (92). Delayed CNS secretion of IL-6 may be a contributing factor in the non-fatal outcome of this particular alphavirus.

When primary astrocyte cultures were infected with the virulent VEE strain, V3000, or the attenuated strain, V3010, IL-6 mRNA was induced to significant levels when compared to uninfected controls (123, Figure 4). No significant differences in IL-6 gene expression levels between the two VEE strains were observed (123). More importantly,

Figure 4. IL-6 gene expression in astrocytes.

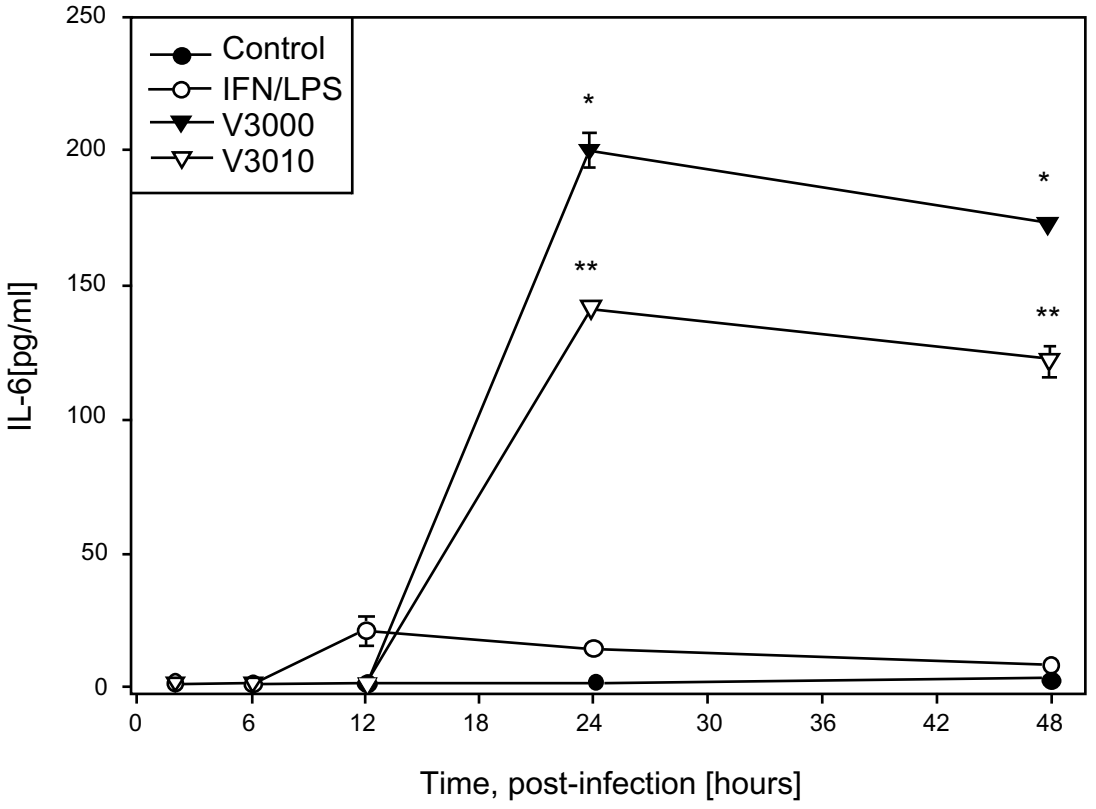


Histograms (mean ± S.E.M. of three independent examples) representing changes in gene expression as measured by PCR amplification and Southern blot analysis for IL-6 based on optical density measurements. Both virulent V3000 and attenuated V3010 induced IL-6 gene expression in primary astrocytes as compared to uninfected controls (*p<0.05). Reproduced from (115), with permission of Dr. Bruce Schoneboom.

when IL-6 protein production was measured in the supernatant of primary astrocyte cultures infected with either the virulent V3000 strain or the attenuated V3010 strain, astrocytes infected with the virulent virus significantly upregulated IL-6 production as compared to both attenuated V3010 infected primary astrocyte cultures and mock-infected controls (123), as seen in Figure 5. Attenuated V3010 infected primary astrocyte cultures upregulated production of IL-6 significantly as compared to primary astrocyte cultures mock-infected with PBS (123). The observation that astrocyte IL-6 mRNA levels following infection with either the virulent V3000 or attenuated V3010 are not significantly different, but astrocyte IL-6 protein levels following infection with either of the two viruses are significantly different indicates possible distinctions in post-transcriptional or post-translational modifications following infection with different VEE strains. This observation further underscores the complexity of responses in the CNS following VEE infection.

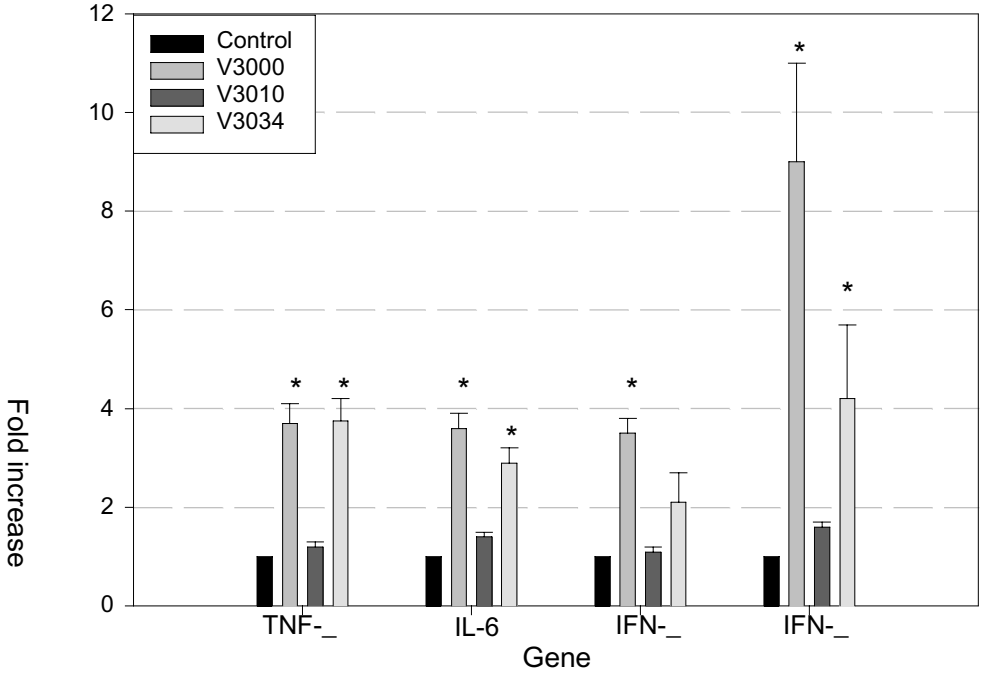
In contrast, adult B6 mice infected with the attenuated V3010 strain did not induce an upregulation of IL-6 mRNA in the whole brain (Figure 6). It is possible that V3010 infection induces only astrocytes to produce this cytokine, and levels of expression are lost when examining the whole environment of the brain. In contrast, similar mice infected with either the virulent V3000 strain or attenuated V3034 strain did show significant IL-6 mRNA expression as compared to control mock-infected mice (124). However, no significant difference in CNS IL-6 mRNA expression was observed between mice infected with the virulent V3000 or the attenuated V3034 (124). Taken together, these results suggest that virulent VEE does induce expression of IL-6 mRNA

Figure 5. IL-6 production in astrocytes.



IL-6 protein was measured by ELISA in the supernatants of primary astrocyte cultures following infection with virulent V3000 or neuro-attenuated V3010. Values expressed as means ± S.E.M. from three independent samples. Infection of astrocytes by both VEE strains resulted in IL-6 secreted in the culture supernatant at 24 and 48 hrs p.i. (*p<0.05), however, neuro-attenuated V3010 infection of astrocytes resulted in lower IL-6 levels as compared to virulent V3000 at both 24 and 48 hrs p.i. (** p<0.05). IFN-γ/LPS-stimulatio of astrocytes resulted in low levels of IL-6 secretion at 12 hrs, but these levels declined and returned to near baseline levels by 48 hrs. Reproduced from (115), with permission of Dr. Bruce Schoneboom.

Figure 6. Pro-inflammatory cytokines gene expression in mice.



Six to 8-week-old female C57BL/6J mice were infected with 1 x 10^3 PFU of one of the VEE strains (attenuated V3010 or V3034, or virulent V3000) via left rear footpad injection. Brains were harvested daily on days 3 - 7 p.i. Total cellular RNA was extracted and then analyzed via RPA. Gene expression was quantitated as a total for the entire experimental time course by measuring pixel densities of autoradiograms from three independent assays (n = 15 mice per group) and determing ratios of inducible genes over the constitutively expressed GAPDH gene and expressed as a relative fold increase (mean ± S.E.M.) as compared to mock-infected controls. Proinflammatory genes were significantly upregulated for all genes tested for V3000, and for TNF-α, IL-6, and IFN-ß for attenuated V3034 (*p<0.05). Reproduced from (116), with permission of Dr. Bruce Schoneboom.

and that differences in IL-6 mRNA expression are observed between two avirulent VEE mutants.

Interferon-β

The cytokines called interferons are so named from their ability to interfere with viral replication. There are three main interferons: IFN-α, IFN-β, and IFN-γ. IFN-α and -β are often referred to as Type 1 interferons. Interferons are not virus-specific, and thus are able to protect against a wide variety of viruses. These cytokines have potent immunomodulatory abilities, that include promotion of or inhibition of the synthesis of antibodies as well as activation of macrophages, natural killer cells, and T cells (74, 93, 103). IFN-β, in addition to its ability to regulate cell-mediated immune responses against viral invaders, regulates non-specific humoral immune responses (64). As is common for interferons, IFN-β activates NK cells. Due to its ability to protect against viruses, IFN-β has been used in the treatment of condyloma acuminata, which is caused by Human Papilloma Virus infection. In addition, this cytokine, in combination with IFN-α, has been utilized to treat chronic active hepatitis B virus infections (71, 110). Finally, recent studies have suggested that IFN-β may be efficacious in relieving some symptoms of Multiple Sclerosis (100).

The pattern of IFN-β expression in B6 mice following infection with VEE strains is similar to results seen in the IL-6 experiments (Figure 6). Following infection with either the virulent V3000 strain of VEE or the neuroattenuated V3034, IFN-β gene expression is significantly increased in adult B6 mice but not significantly increased following infection with the attenuated V3010 strain (124). Although the IFN-β gene induction in

the CNS of mice following infection with virulent V3000 was not significantly increased as compared to similar mice infected with the attenuated V3034 strain, there was a trend toward increased IFN-β gene induction in the CNS of virulent V3000 infected mice. This trend may explain the differences in mortality between mice infected with the virulent VEE strain as compared to the attenuated VEE strains.

Interferon-γ

Both microglia and astrocytes are capable of producing IFN-γ, also known as Type 2 interferon. Furthermore, each cell type expresses IFN-γ receptors to respond to this cytokine (128, 153). IFN-γ, in addition to inducing the expression of MHC Class I and II molecules on both astrocytes and microglia, also induces synthesis of TNF-α in microglia and TNF-α and IL-6 in astrocytes (98, 128, 154). IFN-γ, with its anti-viral and anti-parasitic effects, has also been utilized to treat opportunistic AIDS infections and septic shock (64).

Increased expression of IFN-γ has been implicated in the neuropathogenesis of Multiple Sclerosis (MS). The first clue to its possible role in MS was the finding that IFN-γ is localized near active plaques in the brains of MS patients (128). Although a clear role has not been elucidated, Sei and co-worker s investigations into the role of IFN-γ in MS suggest that this cytokine is involved in neurodegeneration via an inflammatory mechanism (128). In contrast to its role in neuropathogenesis of MS, IFN-γ appears to have a neuroprotective role in HIV dementia. Although AIDS patients without neurological disease display an increased amount of intracerebral IFN-γ mRNA expression, those patients developing HIV dementia have a slowly declining level of

intracerebral IFN-γ mRNA expression that is absent by the onset of opportunistic infections (148). Thus, these data suggest that as potentially protective CNS levels of IFN-γ decrease, patients have an increased likelihood of HIV dementia.

In contrast to the rapid increase of IL-1β and IL-6 mRNA expression observed in the brains of BALB/cJ mice infected with SV, IFN-γ is only slowly induced. Evidence of IFN-γ mRNA expression in the CNS is not seen until day three post-infection. IFN-γ mRNA peaks by day-eight post-infection, and returns to basal levels by day 14 post-infection (149). Immunodeficient SCID mice infected with neurovirulent SV show no detectable levels of IFN-γ mRNA in the CNS (149).

Similar kinetics are seen in the CNS of Biozzi ABH mice infected with SFV. IFN-γ is not detected until day 10 post-infection and never to high levels. However, levels of IFN-γ do remain slightly elevated beyond day 35 post-infection (92). As this infection of mice is non-fatal and asymptomatic, the relatively reduced levels of the inflammatory IFN-γ may actually be beneficial to the host.

Similar results were observed in adult B6 mice infected with either virulent V3000 or attenuated V3010 or V3034. Mice infected with the virulent VEE phenotype displayed a significant increase in IFN-γ mRNA upregulation in the CNS as compared to similar mice infected with either one of the two attenuated strains or mock infected controls (124), and as shown in Figure 6. This significant upregulation of IFN-γ message in the CNS following infection with the virulent strain of VEE may contribute to the difference in outcome of infection as compared to infection with either of the attenuated VEE phenotypes.

Tumor Necrosis Factor-α

Astrocytes, microglia, and some neurons produce TNF-α, which is increased following brain injury (25, 81, 128). TNF-α induces synthesis of IL-6 and activates cell proliferation in astrocytes. In microglia, this cytokine additionally induces synthesis of IL-1β. It is possible that TNF-α mediates and enhances CNS inflammatory responses by increasing permeability of the blood brain barrier and inducing proliferation of astrocytes (128). However, TNF-α is cytotoxic to oligodendrocytes and can induce demyelination (130).

TNF-α has been implicated in a number of neurodegenerative diseases, including MS, Parkinson s Disease, and Creutzfeld-Jacob Disease, that is a type of transmissible subacute spongiform encephalopathy (7, 21, 90, 151). The role of TNF-α in the induction of gliosis and demyelination has been suggested as the mechanism by which neurodegeneration occurs in the above diseases (7). TNF-α is also implicated in the pathogenesis of a variety of infectious diseases that affect the CNS, and include bacterial meningitis, cerebral malaria, and HIV dementia (49, 53, 119, 148). It is interesting to note that giving patients suffering from bacterial meningitis anti-TNF-α antibodies actually improves disease outcome (119), further implicating this cytokine in pathogenesis.

Like IL-1β, TNF-α levels are elevated in HAM/TSP patients, but not in adult T cell leukemia patients who are seropositive for HTLV-1 or asymptomatic carriers (140). TNF-α has also been implicated in the neuropathogenesis of another retroviral phenomenon, HIV dementia. Patients with HIV dementia display significantly elevated

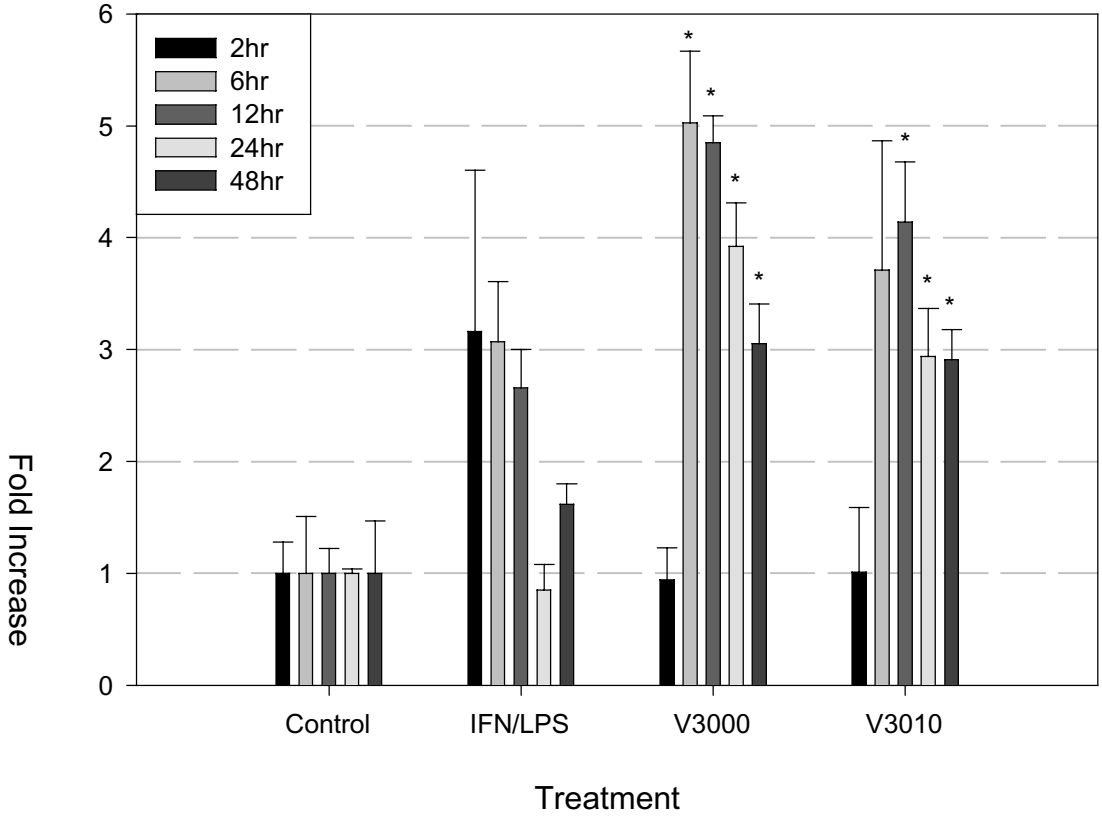
levels of intracerebral TNF-α mRNA, while in AIDS patients displaying no neurological symptoms, no change in intracerebral levels of TNF-α mRNA is observed (148). Collectively, these data strongly support a role for TNF-α in retroviral neurodegeneration.

As in retroviral encephalopathies, TNF-α also plays an important role in the CNS following alphavirus encephalitis. In BALB/cJ mice infected with SV, TNF-α mRNA expression was rapidly upregulated in the CNS. TNF-α mRNA expression is observed within the first 24 hours following infection, peaks at day-eight post-infection, and returnsng to basal levels by day-20 post-infection (149). This phenomenon varies in mouse strains with differnt genetic backgrounds; e.g., the SJL mouse strain is much more susceptible to encephalitis than is the BALB/cJ strain (114). TNF-α mRNA expression in the CNS of SJL mice stays elevated at higher levels, and for a longer time period, following SV infection than in BALB/cJ mice infected with the same virus (114). These data further support the hypothesis that TNF-α contributes to neurotoxicity following alphavirus infection. Also supporting this hypothesis is the observation that SCID mice infected with the neurovirulent strain of SV, NSV, show only the slightest increase in CNS TNF-α mRNA expression but display the best disease outcome and lowest mortality rates of all mouse strains (149). Accordingly, in an *in vitro* study both microglia and astrocytes in culture produced significantly elevated levels of TNF-α following infection with another neurovirulent strain of SV, while microglia and astrocyte cultures infected with a non-neurovirulent strain of SV failed to induce production of TNF-α (17).

Secretion of TNF-α in the CNS of Biozzi ABH mice following infection with the non-fatal SFV does not occur until day seven post-infection. This proinflammatory cytokine is not secreted in high levels until the day 21 following infection, with secretion levels again decreasing by day 28 post-infection. The level of TNF-α is still elevated in the CNS at day 35 post-infection (92). This delayed secretion of TNF-α in the CNS observed following a non-fatal encephalitis may explain the positive outcome in these mice.

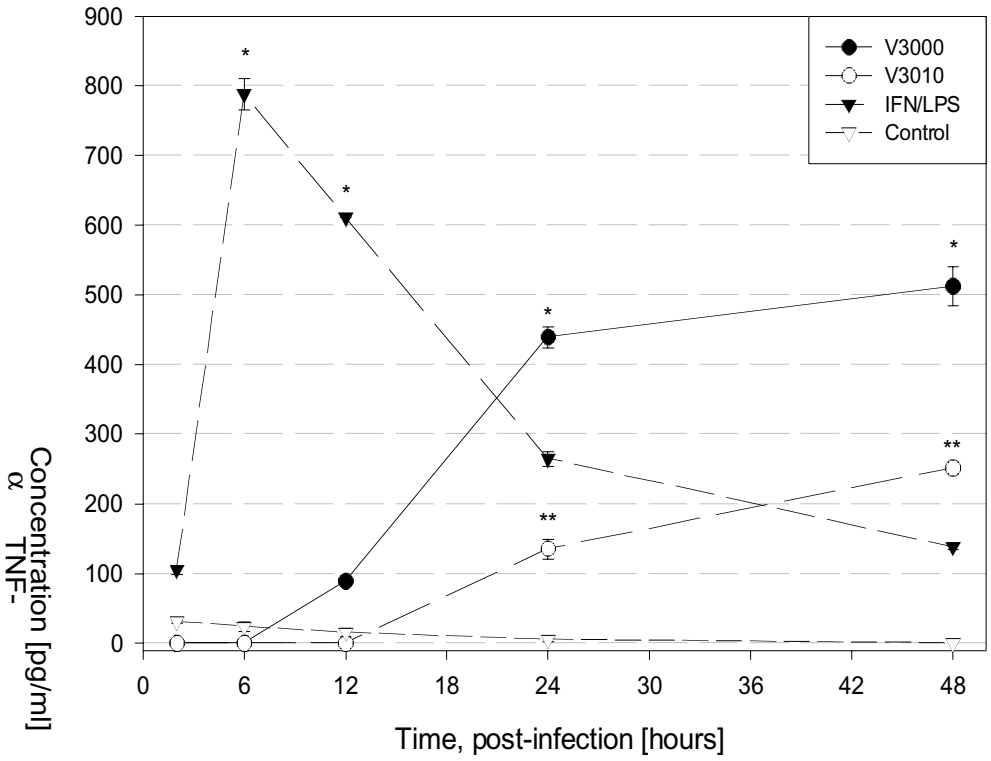
In primary astrocyte cultures infected with either the virulent V3000 strain of VEE or the attenuated V3010, a significant increase in the amount of TNF-α message is observed as compared to mock-infected controls (125). This increase in TNF-α mRNA by primary astrocyte cultures was seen by six hours post-infection in both viral phenotypes (125), as shown in Figure 7. Investigation of the amount of TNF-α secreted by these primary astrocyte cultures in response to infection by VEE produced interesting results. Although astrocyte cultures infected with both viruses displayed increased levels of TNF-α as compared to mock infected primary astrocyte cultures, TNF-α production induced by the virulent V3000 was significantly higher than that induced by the attenuated V3010 (125), Figure 8. These data indicate that both virus strains induce increased TNF-α gene expression in primary astrocyte cultures following infection, but post-transcriptional or post-translational controls may limit the amount of TNF-α actually secreted by astrocytes in response to the attenuated VEE strain. These results could explain the differences observed in mortality associated with each viral phenotype.

Figure 7. TNF-α gene expression in astrocytes.



Histogram (mean ± S.E.M. of three independent samples) depicting changes in gene expression for TNF-α based on optical density measurements in response to VEE infection. Virulent and attenuated VEE significantly induced TNF-α gene expression in primary astrocytes (*p<0.05) as compared to uninfected controls. Virulent V3000 upregulated TNF-α gene expression as early as 6 p.i., and levels of TNF-α gene expression in both virus genotypes were higher as compared to IFN-γ/LPS stimulated astrocytes. Reproduced from (117), with permission of Dr. Bruce Schoneboom.

Figure 8. TNF-α production in astrocytes.



Astrocyte supernatants were analyzed for TNF-α protein levels following infection with virulent V3000 or neuro-attenuated V3010 or stimulated with IFN-γ/LPS. IFN-γ/LPS-stimulated astrocyte production of TNF-α peaked at 6 hrs post stimulation (*p<0.05) and then declined to near baseline levels by 48 hrs. VEE-infected astrocyte production of TNF-α peaked at 48 hr p.i. (*p<0.05). Neuro-attenuated V3010 infection of astrocytes resulted in significantly lower levels of TNF-α as compared to virulent V3000 at 24 and 48 hr p.i. (**p<0.05). Reproduced from (117), with permission of Dr. Bruce Schoneboom.

The results in adult B6 mice were slightly different. Although astrocytes exhibited increased TNF-α message following infection with the attenuated V3010 strain, no detectable increase in TNF-α mRNA expression could be seen in whole mouse brains following infection with the same attenuated strain (124). It is possible that V3010 only induces astrocytes to produce TNF-α following infection, and, in the milieu of the whole brain, this expression is lost. It is interesting that TNF-α gene expression in the brains of mice infected with either the virulent V3000 strain or the attenuated V3034 strain is very similar (124), as shown in Figure 6. Both the virulent V3000 and the attenuated V3034 strain induce TNF-α gene upregulation significantly as compared to mock-infected mice (124). TNF-α s role in the outcome of infection between the various strains of VEE remains elusive; however, previous studies have determined that TNF-receptor knockout mice did display a trend toward increased survival time following infection with either the virulent V3000 or the attenuated V3034 as compared to background strain C57BL/6J mice infected with the same viral phenotypes (124).

Anti-inflammatory Cytokine Responses

Transforming Growth Factor-β

TGF-β is produced by astrocytes, microglia, and oligodendrocytes (128). TGF-β expression appears to be dependent upon the presence of IL-1α, since TGF-β protein can only be detected when elevated IL-1α levels are present (31, 32). TGF-β, in the presence of IL-1α, has been shown to regulate astrocytes and microglia by inhibiting proliferation of astrocytes, inhibiting IFN-γ-induced MHC Class II expression, and inhibiting

microglial synthesis of the proinflammatory cytokines IL-1, IL-6, and TNF-α (128). TGF-β appears to inhibit CNS inflammatory processes and limit neuronal during injury (128). Furthermore, this anti-inflammatory cytokine is crucial in the inhibition of cerebral edema and inflammation (105, 147).

As discussed earlier, TGF-β has been found to be neuroprotective in two experimental models of neurodegenerative diseases, EAE, a model for MS, and EAN, a model for GBS (29). That TGF-β acts as a potent neuroprotective factor is further supported by the observation that MS patients possess decreased levels of TGF-β as compared to normal controls and by the observation that administration of TGF-β ameliorates EAE symptoms in rats (29, 58, 91). Finally, the two approved drugs for the treatment of MS raise levels of TGF-β in MS patients (84).

Additionally, TGF-β appears to be protective in encephalitis induced by Borna disease virus (BDV) (136). BDV is a negative stranded RNA virus of the family *Bornaviridae* which naturally infects horses and causes an often fatal neurological disorder that includes encephalitis (111, 144). BDV has a wide species range which includes the ability to infect and cause fatal encephalitis in sheep, cattle, rabbits, goats, deer, and rhesus monkeys (111). Humans are also susceptible hosts, wherein BDV infection shows a tropism for neurons in the emotional center of the human brain. BDV is now strongly implicated in several human neuropsychiatric disorders including major depression, bipolar disorder, and schizophrenia (14, 48). Interestingly, although immunocompetent hosts develop encephalitis, immunocompromised hosts do not, strongly suggesting that BDV pathology is a virus-induced, cell-mediated

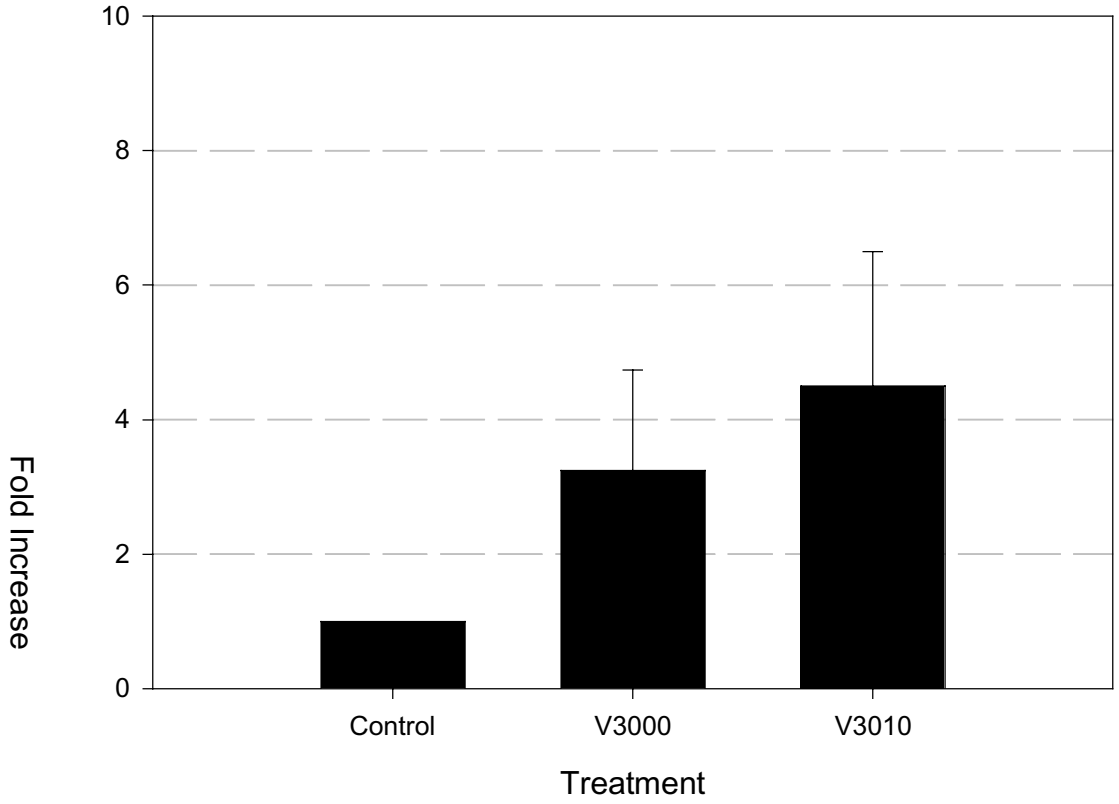
immunopathology (150). TGF-β has been demonstrated to be neuroprotective in BDV infections by delaying this virus-induced immunopathology (136).

If TGF-β is neuroprotective in one encephalitis-inducing virus, it stands to reason that it may be protective in others. In BALB/cJ mice infected with the attenuated TE12 strain of SV, TGF-β mRNA expression in the CNS is comparable to mRNA levels of the pro-inflammatory cytokines IL-1β and IL-6 (149), which suggests that this cytokine may counteract some of the inflammatory effects of these proinflammatory cytokines. Immunodeficient *scid* mice infected with the neurovirulent NSV strain upregulated CNS mRNA expression of TGF-β more than any other cytokine tested (149). Although significant differences in CNS production of pro-inflammatory cytokine message occurred between SV-infected BALB/cJ and SCID mice, no significant differences in the CNS production of TGF-β mRNA was observed (149). The non-fatal outcome of SV-induced encephalitis in *scid* mice may be a combination of the lack of pro-inflammatory cytokines in the CNS and the presence of the anti-inflammatory TGF-β.

Following non-fatal infection of SFV in Biozzi ABH mice, only two cytokines are immediately upregulated: IL-1β and TGF-β (92). TGF-β is secreted by day 3 post-infection and remains present in the CNS until at least day 35 post-infection (92). This observation implies that TGF-β may counteract the inflammatory effects of IL-1β and play a role in the non-fatal outcome of SFV-induced encephalitis.

Following infection with either virulent V3000 or attenuated V3010, primary astrocyte cultures induced expression of upregulated TGF-β mRNA (Figure 9). Although the differences between the treatment groups were not statistically significant, there is a trend whereby astrocyte TGF-β gene expression appears slightly more elevated following

Figure 9. TGF-β gene expression in astrocytes.



Primary rat astrocyte cultures were infected with virulent V3000 or attenuated V3010 at an MOI of 1.0. Total cellular RNA from triplicate samples was collected at 2, 6, 12, 24, and 48 hrs p.i. cDNA was synthesized and amplified via RT-PCR. Amplification products were subjected to gel electrophoresis, transferred to membrane via Southern blot, and visualized via ECL. Autoradiograms were scanned to a digital image. Gene expression was quantitated as a total for the entire experimental time course by measuring pixel densities of autoradiograms from three independent assays and determing ratios of inducible genes over the constitutively expressed GAPDH gene and expressed as a relative fold increase (mean ± S.E.M.) as compared to mock-infected controls. There was no statistical difference between treatment groups.

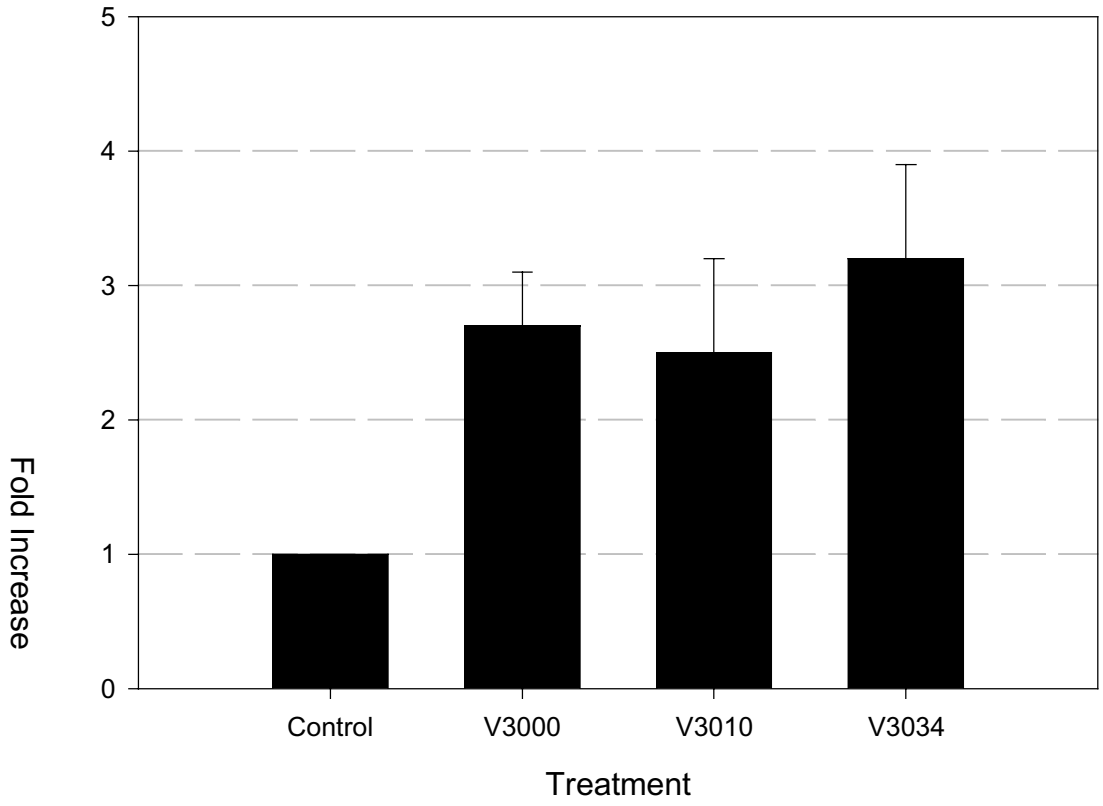
infection with the attenuated V3010 strain when compared to similar astrocytes infected with the virulent V3000 strain. It is possible that the astrocyte upregulation of this anti-inflammatory cytokine following infection with the attenuated VEE strain accounts for the reduced mortality seen in mice infected with this strain of VEE.

Similarly, adult B6 mice infected with either the virulent or one of two attenuated VEE strains upregulated TGF-β gene expression in the CNS (116). As was observed with the primary astrocyte cultures, no significant differences existed between treatment groups (124). However, there again was a trend whereby TGF-β gene expression was slightly upregulated in the CNS in response to the attenuated V3034 strain as compared to the upregulation seen following infection with the virulent V3000 strain (Figure 10). It is unclear why TGF-β gene expression is not upregulated in the CNS of mice following attenuated V3010 infection, and this phenomenon needs to be further investigated. Although the upregulation of TGF-β following infection with VEE is not statistically significant, the trend indicates that this cytokine is probably neuroprotective following infection with VEE, as it is protective in several other CNS disorders.

Interleukin-10

IL-10 is an anti-inflammatory cytokine synthesized by astrocytes and microglia (153). IL-10 inhibits nitric oxide (NO) production and MHC expression in both cell types, while also inhibiting proliferation in microglia (153). IL-10 is able to inhibit the proinflammatory cytokines, including IFN-γ, IL-1β, IL-6, and TNF-α via degradation of their mRNA (64). Interestingly, under normal conditions, microglia produce relatively high amounts of IL-10 and TGF-β as compared to astrocyte production of these same

Figure 10. TGF-β gene expression in mice.



Six to 8-week-old female C57BL/6J mice were infected with 1 x 10^3 PFU of one of the VEE strains (attenuated V3010 or V3034, or virulent V3000) via left rear footpad injection. Brains of 3 mice were harvested daily on days 3 - 7 p.i. Total cellular RNA was extracted and then analyzed via RPA. Gene expression was quantitated as a total for the entire experimental time course by measuring pixel densities of autoradiograms from three independent assays (n = 15 mice per group) and determing ratios of inducible genes over the constitutively expressed GAPDH gene and expressed as a relative fold increase (mean ± S.E.M.) as compared to mock-infected controls. There was no statistical difference between treatment groups.

cytokines, such data strongly suggests the importance of microglia in the maintenance of a normal physiological balance within the CNS (152).

A decrease in IL-10 mRNA expression in the CNS has been implicated in the neuropathogenesis of HIV dementia (148). AIDS patients without neurological disorders have increased amounts of IL-10 message in the CNS as compared to HIV-negative controls. However, patients displaying HIV dementia have decreased levels of IL-10 mRNA in the CNS (148). It would appear that this anti-inflammatory cytokine is somehow neuroprotective and its declining levels are indicative of the development of HIV-associated dementia.

The role of IL-10 in development of SV-induced encephalitis is inconclusive. Immunologically normal BALB/cJ mice infected with the non-fatal TE12 strain of SV displayed a delayed expression of IL-10 message in the CNS. IL-10 mRNA was not seen in the brain until day three post-infection. This mRNA expression peaked at the eighth day following infection, returning to basal levels by day 14 post-infection (149). Immunodeficient SCID mice infected with the neurovirulent strain of SV displayed only a slight increase in CNS IL-10 mRNA levels (149). In another study, SJL mice, a strain more susceptible to encephalitis than BALB/cJ mice, expressed IL-10 mRNA in the CNS to significantly higher levels than did BALB/cJ mice infected with the same neuroattenuated SV strain (114). Additionally, brain lymphocytes isolated from each mouse strain on day 10 post-infection were stimulated *in vitro* and the protein levels of IL-10 were measured via ELISA. Brain lymphocytes from SJL mice secreted high levels of IL-10, while IL-10 was undetectable in cultures from brain lymphocytes from BALB/cJ mice (114). Since BALB/cJ mice are more resistant than SJL mice to SV-

induced encephalitis, it would be expected that the BALB/cJ mice would express higher levels of this anti-inflammatory cytokine. The fact that the data do not conform to expectations illustrates the complexity of the immune response in the CNS. Individual components of the immune system may be measured in response to alphavirus infections, but ultimately, these components influence each other and work in concert in the mileau of the CNS.

Following SFV infection, Biozzi ABH mice secrete IL-10 in the CNS earlier than any other cytokine, except TGF-β and IL-1β (92). IL-10 secretion is very slightly elevated by day three post-infection and peaks by the seventh day following infection. IL-10 production is still elevated at 35 days post-infection (92). The observed early and continued secretion of IL-10 may be protective in this non-fatal viral encephalitis.

The results of IL-10 induction following VEE infection are also inconclusive. Following infection with either the virulent V3000 or one of two attenuated VEE strains, adult B6 mice failed to induce significant gene expression as compared to mock infected controls (124). The role of IL-10 in the neuropathogenesis of VEE infection remains elusive.

Neurotrophic Factor Responses

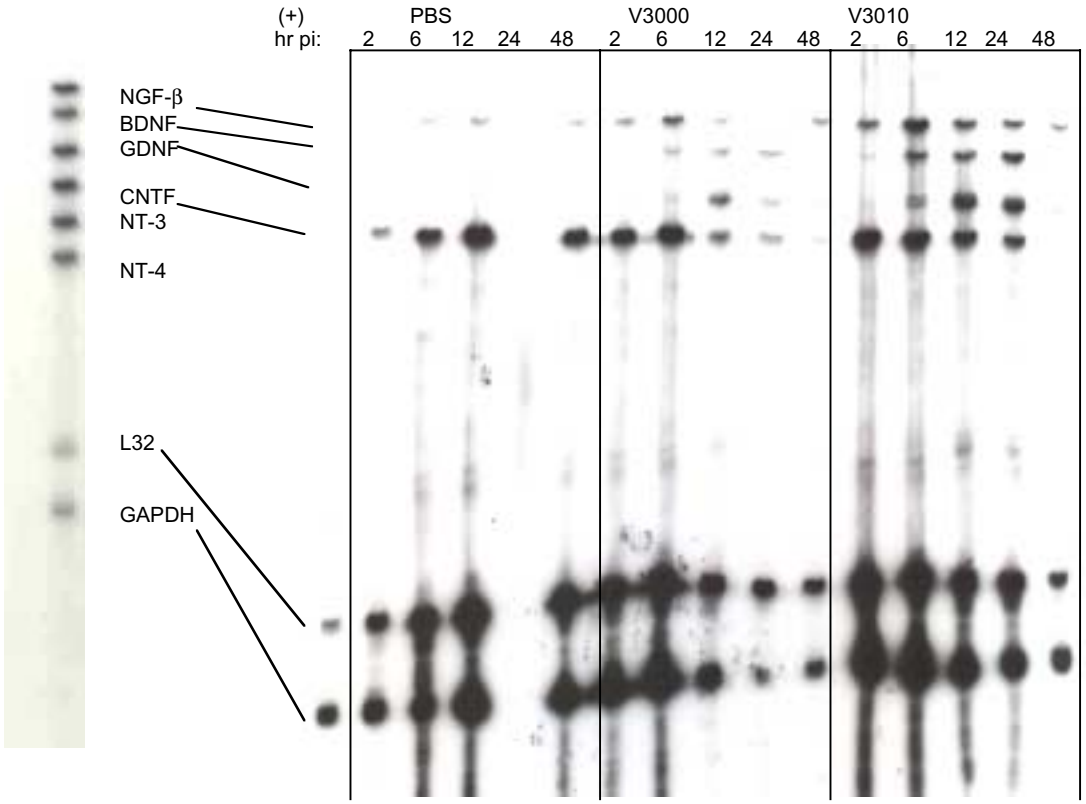
Glial Cell-Line Derived Neurotrophic Factor

Discovered in 1993, GDNF is a potent survival factor for dopaminergic neurons and spinal motor neurons (16, 115). GDNF is 100-times more effective in promoting survival of these types of neurons than other neurotrophic factors (115). Widely expressed in many neuronal and glial tissues, GDNF is distantly related to TGF-β, as evidenced by its

seven cysteine residues which are conserved in all members of the TGF-β superfamily (57, 64). This neurotrophin s ability to inhibit neurodegeneration in Parkinson s disease and provide neuroprotection for dopaminergic and motor neurons has lead to intense research into viability of utilizing GDNF in the treatment of Parkinson s disease (16, 57, 115, 135). Although GDNF increases the survival and differentiation of dopaminergic neurons, it does not increase the total number of neurons or astrocytes. GDNF promotes the uptake of dopamine in dopaminergic neurons, but does not affect serotonin uptake by serotoninergic neurons (64).

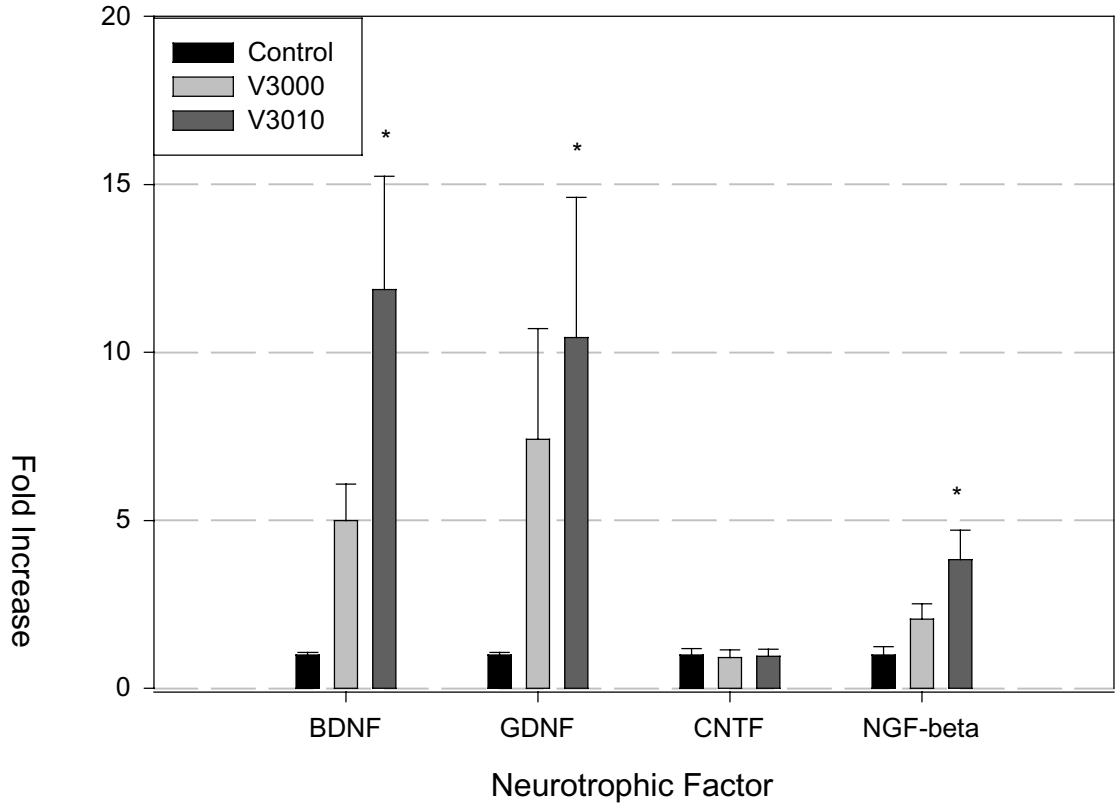
Figure 11 shows the results of an RNase protection assay characterizing the neurotrophin response in astrocytes. When the cultures were infected with either the virulent V3000 strain or the attenuated V3010 strain distinct differences are observed. Primary astrocyte cultures infected with the attenuated strain displayed an increased expression of several neurotrophic factor genes, including GDNF. When these data were re-expressed showing fold increase in mRNA levels, GDNF gene expression in primary astrocyte cultures infected with the attenuated V3010 was significantly upregulated as compared to uninfected controls (124), as seen in Figure 12. Since astrocytes are known to fulfill a support function for neurons, astrocyte neurotrophin response following VEE infection is most likely an important neuroprotective component. The observation that astrocyte neurotrophic support is significantly upregulated following infection with the attenuated VEE strain, but not following infection with the virulent strain, supports the hypothesis that this factor is involved in eliciting different disease outcomes following infection with VEE strains of different neurovirulence.

Figure 11. Autoradiogram of neurotrophin gene expression in astrocytes.



Primary rat astrocytes were infected with V3000 or V3010 at an MOI of 1.0. Total cellular RNA from triplicate samples was collected at 2, 6, 12, 24, and 48 hrs p.i. and analyzed using RNase protection assay (one of three independent assays shown). Controls include: undigested probes serving as size markers, unprotected RNA treated with RNase (RNase digest control), RNA harvested from resting and LPS stimulated macrophages. V3010 infected mice showed an upregulation of neurotrophin genes throughout the 48 hr timecourse as compared to uninfected controls.

Figure 12. Neurotrophin gene expression in astrocytes.



Primary rat astrocytes were infected with V3000 or V3010 at an MOI of 1.0. Total cellular RNA from triplicate samples was collected at 2, 6, 12, 24, and 48 hrs p.i. and analyzed via RPA. Gene expression was quantitated as a total for the entire experimental time course by measuring pixel densities of autoradiograms from three independent assays and determing ratios of inducible genes over the constitutively expressed GAPDH gene and expressed as a relative fold increase (mean ± S.E.M.) as compared to mock-infected controls. Both virulent V3000 and attenuated V3010 upregulated gene expression of GDNF, BDNF, and NGF-β; these were statistically significant for attenuated V3010 (*P<0.05).

Brain Derived Neurotrophic Factor

BDNF is predominantly expressed in neurons of the hippocampus, cortex, and basal forebrain (64). BDNF is selective in its biological activities, primarily supporting survival of primary sensory neurons, but it has been shown to promote survival of other neuron types, such as dopaminergic neurons, certain cholinergic neurons, and spinal motor neurons (27, 64). Although most transgenic mice with a homozygous targeted disruption of the BDNF gene die within a day or two after birth, the few that survive to two to four weeks display nervous system dysfunction and significantly decreased numbers of sensory neurons in the cranium and spine (41, 133). BDNF is important in the development of learning and memory, as well as in synaptic transmission (39, 72). Additionally, BDNF appears to have therapeutic value in the treatment of Alzheimer s Disease, Parkinson s Disease, and Amylotrophic Lateral Scleropathy (27, 64).

The results regarding the upregulation of BDNF gene expression in primary astrocyte cultures infected with VEE are parallel to the results observed with GDNF. Again, BDNF mRNA was significantly upregulated in primary astrocyte cultures infected with the attenuated V3010 strain of VEE as compared to mock infected astrocyte cultures. There was no significant difference in the level of BDNF gene induction in similar astrocytes following infection with the virulent V3000 (124), as is illustrated in Figure 12. Astrocytes, functioning in their critical role of neuronal support, provide a great degree of neuroprotection in response to a variety of CNS injuries. Therefore, it is not surprising that they should provide critical support following infection with VEE.

The upregulation of neurotrophins by astrocytes may be a key component of the immune response to VEE, possibly influencing the ultimate outcome of infection.

Ciliary Neurotrophic Factor

Although CNTF is primarily found in peripheral nerve tissues, astrocytes are a source of CNTF within the CNS (64). In contrast to the role of other neurotrophins during development, CNTF appears to function as a maintenance and repair factor for adult neurons following neuronal injuries (64, 79). A variety of cells, including microglia and oligodendrocytes, respond to CNTF (131), protecting them from apoptosis but not necrosis (64). CNTF knockout mice display normal CNS development, supporting the observation that CNTF does not appear to be expressed during CNS development; however, certain subsets of neurons, particularly motorneurons, are lost postnatally in these transgenic mice (118, 131). These data further support observations that CNTF functions more in maintenance than in development. In an alternative hypothesis, CNTF function may be redundant. CNTF levels in Parkinson s and Alzheimer s patients appear to be lower than normal controls (118). Due to its ability to maintain and repair motor neurons, CNTF may be of particular therapeutic benefit in treating ALS (64).

Primary astrocyte cultures infected with either the virulent V3000 or the attenuated V3010 showed no induction of CNTF gene expression following infection, as illustrated in Figure 12. This is surprising considering CNTF s critical role in repair following injury and infection. It is possible that following VEE infection a cell type other than astrocytes respond with an upregulation of CNTF gene expression, but this explanation is not entirely satisfactory either. Astrocytes are the main source of CNTF in the CNS, and,

therefore, it stands to reason that these cells would be the most likely cell type to respond with an upregulation of this neurotrophic factor. Further investigations to understand this dichotomy are necessary.

Nerve Growth Factor-β

NGF-β functions as a survival factor in the peripheral nervous system, enhancing survival and differentiation of the sensory and sympathetic neurons there. In the CNS, this neurotrophic factor has influence on the development of cholinergic neurons (64). Increased amounts of NGF are seen in inflammatory responses, autoimmune diseases, allergies, and parasitic infections (3). This phenomenon corresponds to the observation that the proinflammatory cytokines IL-1 and IL-6, also seen in increased levels in inflammatory responses, are potent inducers of NGF-β. In astrocytes, IL-1, TNF-α, and TGF-β enhance production of NGF-β (64). The ability of NGF-β to enhance proliferation of mast cells and increase histamine release supports its role in allergic disease (64). In addition to being found within the nervous system, NGF-β has also been discovered in immune organs such as the spleen, thymus, and lymph nodes, and may play a role in activating immune cells (3). This observation has lead to the intense study of NGF as a means of identifying mechanisms implicated in neuroimmune disorders (3).

NGF-β gene induction in primary astrocyte cultures following VEE infection, like that of BDNF and GDNF, displays a significant upregulation in astrocytes following infection with the attenuated VEE strain as compared to mock infected astrocytes, as seen in Figure 12. This gene was not significantly induced in astrocytes following infection with the virulent VEE phenotype. The significant upregulation of this important,

protective neurotrophic factor following infection with the attenuated virus could aid in explaining the difference in mortality seen in the two virus phenotypes.

The CNS cytokine and neurotrophin response to VEE is only part of the broad picture of the CNS immune response to this encephalitic virus. Inflammation, apoptosis, and edema, among other factors, are also very important components of this story. The intricate balance of elements involved in the innate immune response to this virus are elaborate and complex. Much more research needs to be carried out in this area before a full understanding is reached.

DISCUSSION

The complicated interactions in the CNS among resident cells, migrating immune cells, cytokines, and neurotrophins in response to VEE infection represents a complex network. Understanding components of this network and their interactions in response to VEE infection will help elucidate which components contribute to neuropathogenesis and which may protect against neurodegeneration. The ultimate goal of research into the CNS immune response to VEE is to gain insight into these issues and determine which immune events are beneficial to the host and which are neuropathological. With a better understanding, it may be possible to suppress the detrimental reactions and take advantage of the potentially beneficial events to develop treatment modalities for VEE-induced encephalitis.

As presented in this thesis, at least a portion of the immune response in the CNS following infection with VEE appears to contribute to VEE-induced pathogenesis. This is evidenced by the fact that several proinflammatory cytokines, including IL-1β, TNF-α, and IL-6, are significantly upregulated in the CNS of mice and in primary astrocyte cultures infected with virulent VEE (124, 125). In contrast, this significant upregulation of proinflammatory cytokines is generally delayed or absent following infection with attenuated strains of VEE (124, 125). These different cytokine profiles following infection with virulent or attenuated VEE strains cannot be explained merely by the amount of virus present and replicating in the CNS, since all VEE strains are detectable in the CNS by the third day following infection, and all replicate to similar titers (124). Therefore, these data suggest that the differences observed in mortalities of mice after infection with each of these three strains cannot be accounted for by viral presence alone,

but rather, as the result of some specific interactions of virus and target cells, or some other indirect mechanism.

Although astrocyte induction of IL-1β mRNA is not significantly increased following infection with either virulent V3000 or attenuated V3010, there was some increase of IL-1β mRNA in response to both viruses in astrocytes. Although not statistically significant, the virulent V3000 did appear to upregulate IL-1β mRNA in astrocytes as compared to the attenuated V3010. When IL-1β production in astrocytes in response to VEE infection was examined, both strains were able to significantly induce IL-1β production compared to mock-infected controls. Furthermore, the virulent V3000 was able to significantly upregulate IL-1β production in astrocytes when compared to the attenuated V3010. One possible explanation for the dichotomy between non-statistically significant upregulation of IL-1β mRNA followed by a significant upregulation of IL-1β protein could be that the different viruses induce altered post-transcriptional or post-translational modifications. This could be a result of the mutated envelope glycoproteins interacting with the host cell in such a way that cell signaling is modified and transcription or translation are altered as such. IL-1β mRNA production in the brains of mice is significantly upregulated in response to the virulent V3000 when compared to attenuated strains or mock-infected controls. As this proinflammatory cytokine is known to be neurotoxic, it is plausible that the increase seen following infection with the virulent strain could contribute to the higher mortality of the virulent V3000.

The role of IL-6 in VEE pathogenesis is not clear. The data described for VEE-induced astrocyte production of IL-1β mRNA and protein run parallel with the data collected for IL-6 mRNA and protein. Although there is no statistically significant

difference between astrocyte IL-6 mRNA upregulation between the two virus strains, there is a statistical significance in IL-6 protein production between the virulent V3000 and the attenuated V3010, perhaps caused by the same underlying mechanism responsible for the disparity in the IL-β astrocyte response. To add to the complexity, IL-6 mRNA is significantly upregulated in astrocytes in response to attenuated V3010 when compared to mock-infected controls, but not in the brains of mice infected with V3010. One possible explanation for this dissimilarity is that the significant IL-6 mRNA upregulation in astrocytes is lost in the whole environment of the brain. Additionally, IL-6 mRNA expression in the brains of mice is significantly upregulated in response to both the virulent V3000 and another attenuated strain, V3034, indicating that this cytokine has a complex role in the pathogenesis of VEE. As IL-6 is a cytokine with both pro-inflammatory and anti-inflammatory properties, this observation is not surprising.

The mRNA of the proinflammatory cytokines TNF-α, IFN-β, and IFN-γ, was significantly upregulated in the brains of mice infected with virulent V3000 when compared to mock-infected controls. The mRNA of these cytokines were not significantly upregulated following infection with the attenuated V3010. Two of these cytokines, TNF-α and IFN-γ, displayed significant mRNA upregulation following infection with the attenuated V3034. As discussed earlier, V3034 induces a higher mortality rate in mice when compared to V3010. Taken together, these results indicate that the proinflammatory response of these cytokines may contribute to differences in mortality seen in mice in response to the varied VEE strains.

The upregulation of the inflammatory response correlates with a number of other observations seen following CNS infection with VEE. In addition to proinflammatory

cytokines, other inflammatory molecules are upregulated in the CNS in response to VEE infection. Inducible nitric oxide synthase (iNOS) is one such molecule (124). A potent immunomodulatory regulator, iNOS is the enzyme that catalyzes the production of nitric oxide ($NO^{\bullet}$). Although $NO^{\bullet}$ is a powerful defense against invading microbes, it can also be toxic to host cells. In iNOS knockout mice infected with the virulent VEE strain, average survival times were significantly increased as compared to background strain B6 mice infected with the same virus (116); however, mortality rates following virulent VEE infection in these iNOS knockout mice remained unchanged (124). These findings suggest that iNOS may contribute to VEE pathogenesis.

Another consequence of the inflammatory cascade, the occurrence of apoptosis, was also investigated following VEE infection. Apoptosis, or programmed cell death, in response to viral infections, is common and may be particularly detrimental in the environment of the CNS where neurons cannot regenerate readily (3, 76). Apoptosis occurs as a result of VEE infection (67, 124). Although apoptosis does occur in portions of the brain directly infected by the virus (67), further research demonstrated that apoptosis also occurs in areas of the brain where no viral antigen is present (124). Parallel to these findings is the observation that astrogliosis, an inflammatory reaction in the CNS, is also present in areas of the brain where no VEE antigen was detectable (124). The presence of apoptosis and astrogliosis in areas of the brain where VEE infection is not apparent indicates that a mechanism other than direct VEE infection is responsible for at least some of the neuropathological processes and inflammation seen in response to VEE. Additionally, the amount of apoptosis and astrogliosis in the CNS following VEE infection directly correlates with the virulence of the VEE strain; the attenuated strain

displays significantly reduced levels of astrogliosis and apoptotic cells when compared to the virulent strain (124).

Another indication of inflammation, cerebral edema, was also measured in adult B6 mice infected with the virulent V3000 or either of the attenuated strains, V3010 or V3034 (124). Mice infected with the attenuated V3010 strain showed only a marginal increase in total brain weight as a measure of edema when compared to similar mock-infected mice. Although the attenuated V3034-infected mice showed signs of cerebral edema, the measurements were significantly lower than those induced by infection with the virulent strain (124). Taken together, these data imply a correlation among three important factors of CNS inflammation: apoptosis, astrogliosis, and edema (124). All three of these factors are increased following virulent VEE infection as compared to the two attenuated strains. Inflammatory response in the CNS has been implicated in the pathology of a number of viral infections (44, 116, 123), as well as in bacterial and parasitic infections (48, 111), and in neurodegenerative disorders (34, 81, 82, 135). Therefore, it is not surprising that the data support inflammation-mediated pathology following VEE infection.

These findings in VEE pathogenesis correlate well with those from studies performed with other alphaviruses. Infection with Sindbis virus also precipitates a vigorous proinflammatory cytokine response in the CNS, while immunodeficient *scid* mice survive infection with an SV strain that is fatal in normal, immunocompent mice (149). Additionally, the cytokine profile following Semliki Forest virus infection correlates with the severity of disease (92). Increased proinflammatory cytokines are associated with an increased severity of disease following SFV infection. The complex

mechanism of neuropathogenesis among different alphaviruses appear to share key components.

Additionally, the neurotrophic response to attenuated VEE is significantly increased as compared to infection with the virulent VEE strain (124). BDNF, GDNF, and NGF-β are all important components of growth and support for neurons. The observation that these factors are upregulated following infection with VEE indicates that important mechanisms involved in restoration and repair are initiated following virus replication. Furthermore, this significant upregulation of neurotrophins in astrocytes following attenuated V3010 infection may contribute to the beneficial outcome seen in mice infected with this strain as compared to that seen following virulent VEE infection.

The research presented in this thesis was performed to help determine which host responses to VEE are beneficial and which are detrimental, so that future research may take advantage of acquired knowledge and manipulate these responses for therapeutic benefit in the treatment of VEE and other encephalitides. Determining beneficial and detrimental host responses has been advantageous in a number of other infectious diseases. For example, administration of anti-TNF-α- and anti-IL-1-antibodies to patients suffering from bacterial meningitis was shown to be protective and was correlated with recovery (119). Additionally, the administration of neurotrophic factors has also been protective in several neurodegenerative disorders, including Alzheimer s disease and Parkinson s disease (5, 27, 129). Given the neurotrophins role in supporting neurons and preventing apoptosis, administering such agents in an infection characterized by apoptosis has potential future therapeutic applications.

However, additional research needs to be done in the area of VEE neuropathogenesis before these treatment modalities could become a reality. In addition to astroglial response to VEE infection, the role of other resident CNS cells such as microglia must be investigated as well. Further studies into the role of TNF-α in VEE neuropathogenesis are also necessary. Finally, investigating the pattern and outcome of disease following VEE infection in transgenic mice with targeted mutations in cytokine and neurotrophin genes may be utilized to further elucidate the importance of specific cytokines and neurotrophins in response to VEE infection. The complexities involved in VEE-induced neurodegeneration are a fascinating area of research that warrant further investigation.

In conclusion, the neuropathological response to VEE infection cannot be explained exclusively by the presence of virus in the CNS. One or more additional mechanisms must be involved, and the inflammatory response in the CNS has been implicated as one possible mediator. Given the varied cytokine and neurotrophin responses to virulent versus attenuated VEE strains, these responses are strongly implicated as a possible underlying mechanism involved in VEE neuropathogenesis. Further investigation into VEE neuropathogenesis will provide a better understanding of beneficial and detrimental responses in the CNS following VEE infection so that these responses may be manipulated for therapeutic modalities.

REFERENCES

1. Aggoun-Zouaoi, D., C. Charriaut-Marlangue, S. Rivera, I. Jorquera, Y. Ben-Ari, and A. Represa. 1996. The HIV-1 envelope protein gp120 induces neuronal apoptosis in hippocampal slices. *Neuroreport*. 7:433-436.
2. Akira, S., T. Hirano, T. Taga, and T. Kishimoto. 1990. Biology of multifunctional cytokines: IL-6 and related molecules (IL-1 and TNF). *FASEB J*. 4:2860-2867.
3. Aloe, L., L. Bracci-Laudiero, S. Bonini, and L. Manni. 1997. The expanding role of nerve growth factor: from neurotrophic activity to immunologic diseases. *Allergy*. 52:883-894.
4. Altman, L. 1992. Programmed cell death: the paths to suicide. *Trends Neurosci*. 15:278-280.
5. Arenas, E., M. Trupp, P. Akerud, and C. Ibanez. 1995. GDNF prevents degeneration and promotes the phenotype of brain noradrenergic neurons in vivo. *Neuron*. 15:1465-1473.
6. Aronson, J., F. Grieder, N. Davis, P. Charles, T. Knott, K. Brown, and R. Johnston. 2000. A single-site mutant and revertants arising in vivo define early steps in the pathogenesis of Venezuelan equine encephalitis virus. *Virology*. 270:111-123.
7. Arvin, B., L. Neville, F. Barone, and G. Feuerstein. 1996. The role of inflammation and cytokines in brain injury. *Neurosci Behav Rev*. 20:445-452.
8. Bagetta, G., M. Corasaniti, W. Malorni, G. Rainaldi, L. Berliocchi, A. Finazzi-Agro, and G. Nistico. 1996. The HIV-1 gp120 causes ultrastructural changes typical of apoptosis in the rat cerebral cortex. *Neuroreport*. 7:1722-1724.

9. Banati, R., J. Gehrmann, P. Schubert, and G. Kreutzberg. 1993. Cytotoxicity of microglia. *Glia*. 7:111-118.

10. Barone, F., B. Arvin, R. White, A. Miller, C. Webb, R. Willette, P. Lysko, and G. Feuerstein. 1997. Tumor necrosis factor-α. A mediator of focal ischemic brain injury. *Stroke*. 28:1233-1244.

11. Bechmann, I., and R. Nitsch. 1997. Astrocytes and microglial cells incorporate degenerating fibers following entorhinal lesion: a light, confocal, and electron microscopical study using a phagocytosis-dependent labelling technique. *Glia*. 20:145-154.

12. Benjamini, E., G. Sunshine, and S. Leskowitz. 1996. *Immunology: A Short Course*. Wiley-Liss, Inc., New York.

13. Berkedal-Hansen, H. 1993. Role of cytokine and inflammatory mediators in tissue destruction. *J Period Res*. 28:500-510.

14. Bode, L., R. Ferszt, and G. Czech. 1993. Borna disease virus infection and affective disorders in man. *Arch Virol*. 7:159-167.

15. Bodnar, R., C. Pasternak, P. Mann, D. Paul, R. Warren, and D. Donner. 1989. Mediation of anorexia by human recombinant tumor necrosis factor through peripheral action in the rat. *Cancer Res*. 49:6280-6284.

16. Bohn, M. 1999. A commentary on glial cell line-derived neurotrophic factor. *Biochem Pharmacol*. 57:135-142.

17. Brodie, C., N. Weizman, A. Katzoff, A. Lustig, and D. Kobiler. 1997. Astrocyte activation by Sindbis virus: Expression of GFAP, cytokines, and adhesion molecules. *Glia*. 19:275-285.

18. Calisher, C. 1994. Medically important arboviruses of the United States and Canada. *Clin Microbiol Rv*. 7:89-116.

19. Campbell, I. 1998. Structural and functional impact of the transgenic expression of cytokines in the CNS. *Ann NY Acad Sci*. 840:83-96.

20. Campbell, I., C. Abraham, E. Masliah, P. Kemper, J. Inglis, M. Oldstone, and L. Mucke. 1993. Neurologic disease induced in transgenic mice by cerebral overexpression of interleukin-6. *Proc Natl Acad Sci USA*. 90:10061-10065.

21. Campbell, I., M. Eddleston, P. Kemper, M. Oldstone, and M. Hobbs. 1994. Activation of cerebral cytokine gene expression and its correlation with onset of reactive astrocyte and acute-phase response gene expression in scrapie. *J Virol*. 68:2383-2387.

22. Chao, C., S. Hu, and P. Peterson. 1996. Glia: the not so innocent bystanders. *J Neurovirol*. 2:234-239.

23. Charles, P., J. Trgovcich, N. Davis, and R. Johnston. 2000. Immunopathogenesis and immune modulation of Venezuelan equine encephalitis virus-induced disease in the mouse. *J Virol*. in press.

24. Charles, P., E. Walters, F. Margolis, and R. Johnston. 1995. Mechanism of neuroinvasion of Venezuelan equine encephalitis virus in the mouse. *Virology*. 208:662-671.

25. Chung, I., and E. Benveniste. 1990. Tumor necrosis factor-alpha production by astrocytes: Induction by lipopolysaccharide, interferon gamma, and interleukin 1 beta. *J Immunol*. 144:2999-3007.

26. Clarkson, E., W. Zawada, and C. Freed. 1995. GDNF reduces apoptosis in dopaminergic neurons in vitro. *NeuroReport*. 7:145-149.

27. Connor, B., and M. Dragunow. 1998. The role of neuronal growth factors in neurodegenerative disorders of the human brain. *Brain Res Rev*. 27:1-39.

28. Connor, B., D. Young, Q. Yan, R. Faull, B. Synek, and M. Dragunow. 1997. Brain-derived neurotrophic factor is reduced in Alzheimer's disease. *Mol Brain Res*. 49:71-81.

29. Constantinescu, C., B. Hilliard, T. Fujioka, M. Bhopale, D. Calida, and A. Rostami. 1998. Pathogenesis of neuroimmunologic diseases. *Immunol Res*. 17:217-227.

30. Crowley, C., S. Spencer, M. Nishimura, K. Chen, S. Pitts-Meek, M. Armanini, L. Ling, S. McMahon, D. Shelton, A. Levinson, and H. Phillips. 1994. Mice lacking nerve growth factor display perinatal loss of sensory and sympathetic neurons yet develop basal forebrain cholinergic neurons. *Cell*. 76:1001-1011.

31. da Cunha, A., and L. itkovic. 1992. Transforming growth factor-beta 1 expression and regulation in rat cortical astrocytes. *J Neuroimmunol*. 36:157-169.

32. da Cunha, A., J. Jefferson, R. Jackson, and L. Vitkovic. 1993. Glial cell-specific mechanisms of TGF-beta1 induction by IL-1 in cerebral cortex. *J Neuroimmunol*. 42:71-86.

33. Dalrymple, J., S. Schlesinger, and P. Russell. 1976. Antigenic characterization of two Sindbis envelope glycoproteins separated by isoelectric focusing. *Virology*. 69:93-103.

34. Davis, N., F. Grieder, J. Smith, G. Greenwald, M. Valenski, D. Sellon, P. Charles, and R. Johnston. 1994. A molecular genetic approach to the study of Venezuelan equine encephalitis virus pathogenesis. *Arch Virol*. 9:99-109.

35. Davis, N., N. Powell, G. Greenwald, L. Willis, B. Johnson, J. Smith, and R. Johnston. 1991. Attenuating mutations in the E2 glycoprotein gene of Venezuelan equine encephalitis virus: construction of single and multiple mutants in a full-length cDNA clone. *Virology*. 183:20-31.

36. Davis, N., L. Willis, J. Smith, and R. Johnston. 1989. *In vitro* synthesis of infectious Venezuelan equine encephalitis virus RNAfrom a cDNA clone: Analysis of a viable deletion mutant. *Virology*. 171:189-204.

37. del Zoppo, G., I. Ginis, J. Hallenbeck, C. Iadecola, X. Wang, and G. Feuerstein. 2000. Inflammation and stroke: Putative role for cytokines, adhesion molecules and iNOS in brain response to ischemia. *Brain Pathology*. 10:95-112.

38. Donnelly, R., A. Friedhoff, B. Ber, A. Blume, and M. Vitek. 1990. Interleukin-1 stimulates the beta-amyloid precursor protein promoter. *Cell Mol Neurobiol*. 10:485-495.

39. Dragunow, M., E. Beilharz, B. Mason, P. Lawlor, W. Abraham, and P. Gluckman. 1993. Brain-derived neurotrophic factor expression after long-term potentiation. *Neurosci Lett*. 160:232-236.

40. Dubuisson, J., and C. Rice. 1993. Sindbis virus attachment: isolation and characterization of mutants with impaired binding to vertebrate cells. *J Virol*. 67:3363-3374.

41. Ernfors, P., J. Kucera, K. Lee, J. Loring, and R. Jaenisch. 1995. Studies on the physiological role of brain-derived neurotrophic factor and neurotrophin-3 in knockout mice. *Int J Dev Biol*. 39:799-807.

42. Faggioni, R., F. Benigni, and P. Ghezzi. 1995. Pro-inflammatory cytokines as pathogenetic mediators in the central nervous system: Brain-periphery connections. *Neuroimmunomodulation*. 2:2-15.

43. Feuerstein, G., R. Clark, T. Liu, R. White, and F. Barone. 1993. Tumor necrosis factor-alpha mRNA and peptide are markedly expressed in neurons present in focal cerebral stroke (abstract). *Soc Neurosci Abstr*. 19:1656.

44. Feuerstein, G., X. Wang, and F. Barone. 1998. Cytokines in brain ischemia - the role of TNF-α. *Cell Mol Neurobiol*. 18:695-701.

45. Fischer, W., K. Wictorin, A. Bjorklund, L. R. Wiliams, S. Varon, and F. Gage. 1987. Amelioration of cholinergic neuron atrophy and spatial memory impairment in aged rats by nerve growth factor. *Nature*. 329:65-68.

46. Frei, K., U. Malipiero, T. Leist, R. Zinkernagel, M. Schwab, and A. Fontana. 1989. On the cellular source and function of interleukin 6 produced in the central nervous system: Sources, function, and mechanism of action. *Eur J Immunol*. 19:689-694.

47. Frostegard, J., A. Ulfgren, P. Nyberg, U. Hedin, J. Swedenborg, U. Andersson, and G. Hanson. 1999. Cytokine expression in advanced human atherosclerotic plaques: dominance of pro-inflammatory (Th1) and macrophage stimulating cytokines. *Atherosclerosis*. 145:33-43.

48. Fu, Z., J. Amsterdam, M. Kao, V. Shankar, H. Koprowski, and B. Dietzschold. 1993. Detection of Borna disease virus-reactive antibodies from patients with affective disorders by western immunoblot technique. *J Affective Disorders*. 27:61-68.

49. Gallo, P., K. Frei, C. Rordorf, J. Lazdins, B. Tavolato, and A. Fontana. 1989. Human immunodeficiency virus type 1 infection of the central nervous system: An evaluation of cytokines in cerebrospinal fluid. *J Neuroimmunol*. 23:109-116.

50. Gatti, S., and T. Bartfai. 1993. Induction of tumor necrosis factor-alpha mRNA in the brain after peripheral endotoxin treatment: Comparison with interleukin-1 and interleukin-6. *Brain Res*. 624:291-294.

51. Genis, P., M. Jett, E. Bernton, T. Boyle, H. Gelbard, K. Dzenko, R. Keane, L. Resnick, Y. Mizrachi, D. Volsky, L. Epstein, and H. Gendelman. 1992. Cytokines and arachidonic metabolites produced during HIV infected macrophage-astroglia interactions: Implications for the neuropathogenesis of HIV diseases. *J Exp Med*. 176:1703-1718.

52. Giulian, D., T. Baker, L. Shih, and L. Lachman. 1986. Interleukin 1 of the central nervous system is produced by ameboid microglia. *J Exp Med*. 164:594-604.

53. Grau, G., L. Fajardo, P. Piguet, B. Allet, P. Lambert, and P. Vassalli. 1987. Tumor necrosis factor (cachectin) as an essential mediator in murine cerebral malaria. *Science*. 237:210-213.

54. Grieder, F., B. Davis, X. Zhou, S. Chen, F. Finkelman, and W. Gause. 1997. Kinetics of cytokine expression and regulation of host protection following infection with molecularly cloned Venezuelan equine encephalitis virus. *Virology*. 233:302-312.

55. Grieder, F., N. Davis, J. Aronson, P. Charles, D. Sellon, K. Suzuki, and R. Johnston. 1995. Specific restrictions in the progression of Venezuelan equine encephalitis virus-induced disease resulting from single amino acid changes in the glycoproteins. *Virology*. 206:994-1006.

56. Griffin, D. 1997. Cytokines in the brain during viral infection: clues to HIV-associated dementia. *J Clin Invest*. 100:2948-2951.

57. Grondin, R., and G. DM. 1998. Glial cell line-derived neurotrophic factor: a drug candidate for the treatment of Parkinson's disease. *J Neurol*. 245:P35-42.

58. Hafler, D., and H. Weiner. 1989. MS: a CNS and systemic autoimmune disease. *Immunol Today*. 10:104-107.

59. Heese, K., C. Hock, and U. Otten. 1998. Inflammatory signals induce neurotrophin expression in human microglial cells. *J Neurochem*. 70:699-707.

60. Hellweg, R., S. v. Richthofen, D. Anders, C. Baethge, S. Ropke, H. Hartung, and C. Gericke. 1998. The time course of nerve growth factor content in different neuropsychiatric diseases: a unifying hypothesis. *J Neural Transm*. 105:871-903.

61. Henderson, C. 1996. Role of neurotrophic factors in neuronal development. *Curr Opin Neurobiol*. 6:64-70.

62. Higgins, G., and J. Olschowka. 1991. Induction of interleukin 1beta mRNA in adult rat brain. *Mol Brain Res*. 9:143-148.

63. Hommes, D., and S. v. Deventer. 2000. Anti and proinfammatory cytokines in the pathogenesis of tissue damage in Crohn's disease. *Curr Opin Clin Nutr Metab*. 3:191-195.

64. Ibelgaufts, H. 1999. *Cope with Cytokines.* Worldwide Website. www.copewithcytokines.de/cope.cgi.

65. Iwasaki, Y. 1990. Pathology of chronic myelopathy associated with HTLV-1 infection (HAM/TSP). *J Neurol Sci*. 96:103-123.

66. Jackson, A., S. S. Gupta, and J. Smith. 1991. Pathogenesis of Venezuelan equine encephalitis virus infection in mice and hamsters. *Vet Pathol*. 28:410-418.

67. Jackson, A., and J. Rossiter. 1997. Apoptotic cell death is an important cause of neuronal injury in experimental Venezuelan equine encephalitis virus infection of mice. *Acta Neuropathol*. 93:349-353.

68. Janis, L., M. Glasier, G. Martin, R. Stackman, T. Walsh, and D. Stein. 1995. A single intraseptal injection of nerve growth factor facilitates radial maze performance following damage to the medial septum in rats. *Brain Res*. 679:99-109.

69. Johnson, E., J. Chang, T. Koike, and D. Martin. 1989. Why do neurons die when deprived of trophic factors? *Neurobiol Aging*. 10:549-552.

70. Johnston, R., and J. Smith. 1988. Selection for accelerated penetration in cell culture coselects for attenuated mutants of Venezuelan equine encephalitis virus. *Virology*. 162.

71. Kagawa, T., T. Morizane, H. Saito, S. Tsunematsu, S. Tada, N. Kumagai, K. Tsuchimoto, H. Sugiura, M. Mukai, and M. Tsuchiya. 1993. A pilot study of long-term weekly interferon-beta administration for chronic hepatitis B. *Am J Gastroenterol*. 88:212-216.

72. Kang, H., and E. Schuman. 1995. Long-lasting neurotrophin-induced enhancement of synaptic transmission in the adult hippocampus. *Science*. 267:1658-1662.

73. Katila, H., B. Appelberg, M. Hurme, and R. Rimon. 1994. Plasma levels of interleukin-1beta and interleukin 6 in schizophrenia, other psychoses, and affective disorders. *Schizophr Res*. 12:29-34.

74. Kerr, I., and G. Stark. 1992. The antiviral effects of interferons and their inhibition. *J Interferon Res*. 21:237-240.

75. Kimelberg, H., and M. Norenberg. 1989. Astrocytes. *Scientific American*. 260:66-76.

76. Kinney, R., B. Johnson, J. Welch, K. Tsuchiya, and D. Trent. 1989. The full length nucleotide sequence of the virulent Trinidad donkey strain of Venezuelan equine encephalitis virus and its attenuated derivative, strain TC-83. *Virology*. 170:19-30.

77. Kubes, V., and F. Rios. 1939. The causative agent of infectious equine encephalomyelitis in Venezuela. *Science*. 90:20-21.

78. Kuriyama, K., T. Hori, T. Mori, and T. Nakashima. 1990. Actions of interferon-alpha and interleukin 1-beta on glucose responsive neurons in the ventromedial hypothalamus. *Brain Res Bull*. 24:803-810.

79. Kuzis, K., and F. Eckenstein. 1996. Ciliary neurotrophic factor as a motor neuron trophic factor. *Perspect Dev Neurobiol*. 4:65-74.

80. Levine, B., and D. Griffin. 1993. Molecular analysis of neurovirulent strains of Sindbis virus that evolve during persistent infection of scid mice. *J Virol*. 67:6872-6875.

81. Lieberman, A., P. Pitha, H. Shin, and M. Shin. 1989. Production of tumor necrosis factor and other cytokines by astrocytes stimulated with lipopolysaccharide or a neurotropic virus. *Proc Natl Acad Sci USA*. 86:6348-6352.

82. Loo, D., A. Copani, C. Pike, E. Whitemore, A. Walencewicz, and C. Cotman. 1993. Apoptosis is induced by beta amyloid in cultured central nervous system neurons. *Proc Natl Acad Sci USA*. 90:7951-7955.

83. Martiney, J., C. Cuff, M. Litwak, B. J, and B. CF. 1998. Cytokine induced inflammation in the central nervous system revisited. *Neurochem*. 23:349-359.

84. Martino, G., P. Poliani, R. Furlan, P. Marconi, J. Glorioso, L. Adorini, and G. Comi. 2000. Cytokine therapy in immune-mediated demyelinating diseases of the central nervous system: a novel gene therapy approach. *J Neuroimmunol*. 107:184-190.

85. Matsuyma, T., N. Kobayashi, and N. Yamamoto. 1991. Cytokines and HIV infection: is AIDS a tumor necrosis factor disease? *AIDS*. 5:1405-1417.

86. Merrill, J., Y. Koyanagi, and I. Chen. 1991. HIV-1, macrophages, glial cells, and cytokines in AIDS nervous system disease. *FASEB J*. 5:2391-2397.

87. Mittleman, B., F. Castellanos, L. Jacobsen, J. Rapoport, S. Swedo, and G. Shearer. 1997. Cerebrospinal fluid cytokines in pediatric neuropsychiatric disease. *J Immunol*. 159:2994-2999.

88. MMWR. 1995. Venezuelan equine encephalitis virus - Columbia, 1995. *Morb Mortal Wkly Rep*. 44:721-724.

89. Mogi, M., M. Harada, P. Riederer, H. Inagaki, M. Minami, and T. Nagatsu. 1994. IL-1beta, IL-6, epidermal growth factor and transforming growth factor-alpha are elevated in the brains from Parkinsonian patients. *Neurosi Lett*. 180:147-150.

90. Mogi, M., M. Haruda, P. Riederer, H. Narabayashi, K. Fujita, and T. Nagatsu. 1994. Tumor necrosis factor alpha increases in the brain and cerebrospinal fluis from Parkinsonian patients. *Neurosci Lett*. 165:208-210.

91. Mokhtarian, F., Y. Shi, D. Shirazian, L. Morgante, A. Miller, and D. Grob. 1994. Defective production of anti-inflammatory cytokine TGF-beta by T-cell lines of patients with active multiple sclerosis. *J Immunol*. 152:6003-6010.

92. Morris, M., H. Dyson, D. Baker, L. Harbige, J. Fazakerley, and S. Amor. 1997. Characterization of the cellular and cytokine response in the central nervous system following Semliki Forest virus infection. *J Neuroimmunol*. 74:185-197.

93. Murray, H. 1992. The interferons, macrophage activation, and host defense against nonviral pathogens. *J Interferon Res*. 12:319-322.

94. Murray, K., C. Gall, E. Jones, and P. Isackson. 1994. Differential regulation of brain-derived neurotrophic factor and type II calcium/calmodulin-dependent protein kinase messenger RNA expression in Alzheimer's disease. *Neuroscience*. 60:37-48.

95. Nuoro, G., F. Gallery, P. MacConnell, and A. Brown. 1994. In situ detection of PCR amplified HIV-1 nucleic acids and tumoer necrosis factor-alpha mRNA in the central nervous system. *Am J Pathol*. 144:659-666.

96. Okuda, Y., S. Sakoda, C. Bernard, and T. Yanagihara. 1998. The development of autoimmune encephalomyelitis provoked by myelin oligodendrocyte glycoprotein is associated with an upregulation of both proinflammatory and immunoregulatory cytokines in the central nervous system. *J Int Cyt Res*. 18:415-421.

97. Oppenheim, R. 1991. Cell death during development of the nervous system. *Annu Rev Neurosci*. 14:453-501.

98. Otero, G., and J. Merrill. 1994. Cytokine receptors on glial cells. *Glia*. 11:117-128.

99. Owens, T., T. Renno, V. Taupin, and M. Krakowski. 1994. Inflammatory cytokines in the brain: does the CNS shape immune responses? *Immunol Today*. 15:566-571.

100. Panitch, H. 1992. Interferons in multiple sclerosis: a review of the evidence. *Drugs*. 44:946-962.

101. Pedersen, C. J., and G. Eddy. 1974. Separation, isolation, and immunological studies of Venezuelan equine encephalomyelitis virus. *J Virol*. 14:740-744.

102. Perez-Navarro, E., E. Arenas, J. Reiriz, N. Calvo, and J. Alberch. 1996. Glial cell line-derived neurotrophic factor protects striatal calbindin-immunoreactive neurons from excitotoxic damage. *Neuroscience*. 75:345-352.

103. Pestka, S., J. Langer, K. Zoon, and C. Samuel. 1987. Interferons and their actions. *Ann Rev Biochem*. 56:727-777.

104. Peters, C., and J. Dalrymple. 1990. Alphaviruses, p. 713-761. *In* B. Fields and D. Crupe, et al. (ed.), Virology, 2nd ed. Raven Press, New York.

105. Pfister, H., K. Frie, B. Ottnad, U. Koedel, A. Tomasz, and A. Montana. 1992. Transforming growth factor-beta 2 inhibits cerebrovascular changes and brain edema formation in the tumor necrosis factor alpha-independent early phase of experimental pneumococcal meningitis. *J Exp Med*. 176:265-268.

106. Phillips, H., J. Hains, M. Armanini, G. Laramee, S. Johnson, and J. Winslow. 1991. BDNF mRNA is decreased in the hippocampus of individuals with Alzheimer's disease. *Neuron*. 7:695-702.

107. Plata-Salaman, C., Y. Oomur, and Y. Kai. 1988. Tumor necrosis factor and interleukin-1 beta: Suppression of food intake by direct action in the central nervous system. *Brain Res*. 448:106-114.

108. Randall, R., and J. Mills. 1944. Fatal encephalitis in man due to the Venezuelan virus of equine encephalomyelitis in Trinidad. *Science*. 99:225-226.

109. Regis, E. 1999. *The Biology of Doom: The History of America's Secret Germ Warfare Project*. Henry Holt and Company, New York.

110. Resta, L., M. Troia, S. Russo, G. Colucci, R. Sabatini, L. Loiudice, and G. Cagnazzo. 1992. Variations of lymphocyte sub-populations in vulvar condylomata during therapy with beta-interferon. *Eur J Gynaecol Oncol*. 13:440-444.

111. Richt, J., I. Pfeuffer, M. Christ, K. Frese, K. Bechter, and S. Herzog. 1997. Borna disease virus infection in animals and humans. *Emerg Inf Dis*. 3:343-352.

112. Roerhig, J., and J. Matthews. 1985. The neutralization site on the E2 glycoprotein of Venezuelan equine encephalomyelitis (TC-83) virus is composed of multiple conformationally stable isotopes. *Virology*. 142:347-356.

113. Rothwell, N., and J. Ruton. 1993. Involvement of interleukin-1 and lipocortin-1 in ischemic brain damage. *Cereb and Brain Metab Rev*. 5:178-198.

114. Rowell, J., and D. Griffin. 1999. The inflammatory response to nonfatal Sindbis virus infection of the nervous system is more severe in SJL than in BALB/c mice and is associated with low levels of IL-4 mRNA and high levels of IL-10 producing $CD4^+$ T cells. *J Immunol*. 162:1624-1632.

115. Saarma, M., and H. Sariola. 1999. Other neurotrophic factors: glial cell line-derived neurotrophic factor. *Microsc Res Tech*. 45:292-302.

116. Saffran, B. 1992. Should intracerebroventricular nerve growth factor be used to treat Alzheimer's disease?. *Perspect Biol Med*. 35:471-486.

117. Saper, C., and C. Breder. 1994. The neurologic basis of fever. *N Engl J Med*. 330:1880-1886.

118. Sariola, H., K. Saino, U. Arumae, and M. Saarma. 1994. Neurotrophins and ciliary neurotrophic factor: their biology and pathology. *Ann Med*. 26:355-363.

119. Saukkonen, K., S. Sande, C. Cioffe, S. Wolpe, B. Sherry, A. Cerami, and E. Tuomanen. 1971. The role of cytokines in the generation of inflammation and tissue damage in experimental gram positive meningitis. *J Exp Med*. 171:439-448.

120. Schaar, D., B. Sieber, C. Dreyfus, and I. Black. 1993. Regional and cell-specific expression of GDNF in rat brain. *Exp Neurol*. 124:368-371.

121. Scherer, W., C. Ellsworth, and A. Ventura. 1971. Studies of viral virulence.II. Growth and adsorption curves of virulent and attenuated strains of Venezuelan encephalitis virus in cultured cells. *Am J Pathol*. 62:211-219.

122. Schlesinger, M., S. Schlesinger, and B. Burge. 1972. Identification of a second glycoprotein in Sindbis virus. *Virology*. 47:539-541.

123. Schoneboom, B. 2000. Neuro-immune mechanisms in response to Venezuelan equine encephalitis virus infection. Doctoral dissertation. Uniformed Services University of the Health Sciences, Bethesda, MD.

124. Schoneboom, B., K. Catlin, A. Marty, and F. Grieder. 2000. Inflammation is a component of neurodegeneration in response to Venezuelan equine encephalitis virus in mice. *J Neuroimmunol*. 109:132-146.

125. Schoneboom, B., M. Fultz, T. Miller, L. McKinney, and F. Grieder. 1999. Astrocytes as targets for Venezuelan equine encephalitis virus infection. *J Neurovirol*. 5:342-354.

126. Schoneboom, B., J. Lee, and F. Grieder. 2000. Early expression of interferon alpha and beta and iNOS in the brains of Venezuelan equine encephalitis virus-infected mice. *J Int Cyt Res*. 20:205-215.

127. Scorziello, A., T. Florio, A. Bajetto, and G. Schettini. 1998. Intracellular signalling mediating HIV-1 gp120 neurotoxicity. *Cell Signal*. 10:75-84.

128. Sei, Y., L. Vitkovic, and M. Yokoyama. 1995. Cytokines in the central nervous system: Regulatory roles in neuronal function, cell death, and repair. *Neuroimmunomodulation*. 2:121-133.

129. Seiger, A., A. Nordberg, H. v. Holst, L. Backman, T. Ebendal, I. Alafuzoff, K. Amberla, P. Hartvig, A. Herlitz, A. Lilja, H. Lundqvist, B. Langstrom, B. Meyerson, A. Persson, M. Viitanen, B. Winblad, and L. Olson. 1993. Intracranial infusion of purified nerve growth factor to an Alzheimer patient: the first attempt of a possible future treatment strategy. *Behav Brain Res*. 57:255-261.

130. Selmaj, K., and C. Raine. 1988. Tumor necrosis factor mediates myelin and oligodendrocyte damage in vitro. *Ann Neurol*. 23:339-346.

131. Sendtner, M., P. Carroll, B. Holtmann, R. Hughes, and H. Thoenen. 1994. Ciliary neurotrophic factor. *J Neurobiol*. 25:1436-1453.

132. Singh, A., R. Kulshreshtha, and A. Mathur. 2000. Secretion of the cytokine interleukin-8 during Japanese Encehalitis Virus infection. *J Med Microbiol*. 49:607-612.

133. Snider, W. 1994. Functions of the neurotrophins during nervous system development: what the knockouts are teaching us. *Cell*. 77:627-638.

134. Southern, E. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J Mol Biol*. 98:503-517.

135. Springer, J., L. Bergmann, X. Mu, and J. Trojanowski. 1994. Expression of GDNF mRNA in rat and human nervous tissue. *Exp Neurol*. 127:167-170.

136. Stitz, L., O. Planz, T. Bilzer, K. Frei, and A. Fontana. 1991. Transforming growth factor-beta modulates T cell-mediated encephalitis caused by Borna disease virus: pathogenic importance of $CD8^+$ cells and suppression of antibody formation. *J Immunol*. 147.

137. Strauss, J., B. Pfefferkorn, and J. Darnell. 1969. Identification of the membrane protein and core protein of Sindbis virus. *Proc Natl Acad Sci US*. 59:533-537.

138. Streit, W., and C. Kincaid-Colton. 1995. The brain's immune system. *Scientific American*. 273:54-61.

139. Svanborg, C., G. Godaly, and M. Hedlund. 1999. Cytokine responses during mucosal infections: role in disease pathogenesis and host defense. *Curr Opin Microbiol*. 2:99-105.

140. Tendler, C., S. Greenberg, J. Burton, D. Danielpour, S. Kim, W. Blattner, A. Manns, and T. Waldman. 1991. Cytokine induction of HTLV-1 associated myelopathy and adult T cell leukemia: Alternate molecular mechanisms underlying retroviral pathogenesis. *J Cell Biochem*. 46:302-311.

141. Toggas, S., E. Masliah, E. Rockenstein, G. Rall, C. Abraham, and L. Mucke. 1994. Central nervous system damage produced by expression of HIV-1 coat protein gp120 in transgenic mice. *Nature*. 367:188-193.

142. Toulmond, S., P. Parnet, and A. C. E. Linthorst. 1996. When cytokines get on your nerves: cytokine networks and CNS pathologies. *Trends Neurosci*. 19:409-410.

143. U.S. Army Medical Research Institute of Infectious Diseases. 1996. *Medical Management of Biological Casualties*. Fort Detrick, Frederick, MD.

144. van Regenmortel, M., C. Fauquet, D. Bishop, E. Carstens, M. Estes, S. Lemon, J. Maniloff, M. Mayo, D. McGeoch, C. Pringle, and R. Wickner. 2000. *Virus Taxonomy: The Classification and Nomenclature of Viruses. The Seventh Report of the International Committee on Taxonomy of Viruses*. Academic Press, San Diego.

145. Vandenabeele, P., and W. Fiers. 1991. Is amyloidogenesis during Alzheimer's disease due to an IL-1/IL-6 mediated 'acute phase response' in the brain?*Immunol Today*. 12:217-219.

146. Wahl, S., J. Allen, N. McCartney-Francis, M. Morganti-Kossmann, T. Kossman, L. Ellingsworth, U. Mai, S. Mergenhagen, and J. Orenstein. 1991. Macrophage- and astrocyte-derived transforming growth factor beta as a mediator of central nervous system dysfunction in Acquired Immune Deficiency Syndrome. *J Exp Med*. 173:981-991.

147. Wahl, S., N. McCartney-Francis, and S. Mergenhagen. 1989. Inflammatory and immunomodulatory roles of TGF-beta. *Immunol Today*. 10:28-261.

148. Wesselingh, S., J. Glass, J. McArthur, J. Griffin, and D. Griffin. 1994. Cytokine dysregulation in HIV-associated neurological disease. *Adv Neuroimmunol*. 4:199-206.

149. Wesselingh, S., B. Levine, R. Fox, S. Choi, and D. Griffin. 1994. Intracerebral cytokine mRNA expression during fatal and nonfatal alphavirus encephalitis suggests a predominant type 2 T cell response. *J Immunol*. 152:1289-1297.

150. Williams, B. 1998. *Borna virus suspected cause for neuropsychiatric disorders in humans.* Worldwide Website. www.doofus.org/mercatroid/borna.html.

151. Woodroofe, M. 1995. Cytokine production in the central nervous system. *Neurology*. 45 (Suppl 6):S6-S10.

152. Xiao, B., X. Bai, G. Zhang, B. Hojeberg, and H. Link. 1996. Shift from anti- to proinflammatory cytokine profiles in microglia through LPS or IFN-gamma mediated pathways. *NeuroReport*. 7:1893-1898.

153. Xiao, B., and H. Link. 1998. Immune regulation within the central nervous system. *J Neuro Sci*. 157:1-12.

154. Yong, V., R. Moumdjian, F. Young, T. Ruijus, M. Feedmen, N. Cashman, and J. Antel. 1991. Gamma interferon promotes proliferation of adult human astrocytes in vitro and reactive gliosis in the adult mouse brain in vivo. *Proc Natl Acad Sci USA*. 88:7016-7020.

155. Young, N., and K. Johnson. 1969. Antigenic varients of Venezuelan equine encephalitis virus: their geographic distribution and epidemiological significance. *Am J Epidemiol*. 89:286-307.

156. Zhao, B., and J. Schwartz. 1998. Involvement of cytokines in normal CNS development and neurological diseases: recent progress and perspectives. *J Neurosci Res*. 52:7-16.