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14. ABSTRACT
**Venezuelan equine encephalitis (VEE) virus infects horses and humans and is a potential biothreat pathogen. Agents that alter the blood-brain barrier (BBB) may enhance viral encephalitides. The current studies explored whether tunicamycin (TM) and other agents affect the pathogenesis of VEE. Following infection with the molecularly-cloned virulent V3000 virus TM-treated mice had a significantly ($p < .05$) decreased mean survival time (MST) of 7.3 days versus 9.9 days in controls. Using plaque assay, V3000 reached nearly 107 pfu/gram in the brains of TM-treated mice at 48 hours post inoculation (PI) versus 103 pfu/gram in controls. The attenuated V3034 strain invaded the brains of TM-treated mice by 48 hours PI versus 72 hours for controls and TM-treated mice had 100-fold or more virus at all times. TM-treated and control mice had similar viremia profiles with both viruses and similar V3000 brain replication curves. Immunohistochemistry showed that V3000 invaded the brains of TM-treated mice by 24 hours, versus 48 hours for the controls. V3000 appeared to enter the brain via the olfactory tract, not via the BBB, in both groups. TM-treated mice with V3000 exhibited earlier clinical signs and greater weight loss than controls. The brains of TM-treated mice had earlier and more severe inflammation, neuronal damage, and extravascular fibrinogen and upregulation of several cytokines. Electron microscopy of the brains of uninfected, TM-treated mice revealed perivascular edema and swollen astrocyte endfeet. Additional studies failed to show that pyridostigmine, LPS or TNF-_ significantly altered VEE. Pyridostigmine and LPS-treated mice with V3034 showed similar neuroinvasion patterns as controls and those with V3000 showed similar MSTs as controls. Serum TNF-_ was increased in V3000-infected mice (peak - 52.9 pg/ml at 24 hours). However, mice given antibodies to TNF-_ had identical neuroinvasion patterns as controls. These findings indicate that TM enhances neuroinvasion and pathogenicity in mice infected with VEE viruses but fail to indicate that pyridostigmine, LPS and TNF-_ affect VEE similarly, suggesting TM is unique in its effect on VEE virus. Tunicamycin could be a useful tool in further studies of VEE virus neuroinvasion.**

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**ABSTRACT**

Tunicamycin Enhances Neuroinvasion and Pathogenicity in Mice with Venezuelan Equine Encephalitis Virus

Keith E. Steele, 2003

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Venezuelan equine encephalitis (VEE) virus infects horses and humans and is a potential biothreat pathogen. Agents that alter the blood-brain barrier (BBB) may enhance viral encephalitides. The current studies explored whether tunicamycin (TM) and other agents affect the pathogenesis of VEE.

Following infection with the molecularly-cloned virulent V3000 virus TM-treated mice had a significantly ($p < .05$) decreased mean survival time (MST) of 7.3 days versus 9.9 days in controls. Using plaque assay, V3000 reached nearly $10^7$ pfu/gram in the brains of TM-treated mice at 48 hours post inoculation (PI) versus $10^3$ pfu/gram in controls. The attenuated V3034 strain invaded the brains of TM-treated mice by 48 hours PI versus 72 hours for controls and TM-treated mice had 100-fold or more virus at all times. TM-treated and control mice had similar viremia profiles with both viruses and similar V3000 brain replication curves. Immunohistochemistry showed that V3000 invaded the brains of TM-treated mice by 24 hours, versus 48 hours for the controls. V3000

appeared to enter the brain via the olfactory tract, not via the BBB, in both groups. TM-treated mice with V3000 exhibited earlier clinical signs and greater weight loss than controls. The brains of TM-treated mice had earlier and more severe inflammation, neuronal damage, and extravascular fibrinogen and upregulation of several cytokines. Electron microscopy of the brains of uninfected, TM-treated mice revealed perivascular edema and swollen astrocyte endfeet.

Additional studies failed to show that pyridostigmine, LPS or TNF-_ significantly altered VEE. Pyridostigmine and LPS-treated mice with V3034 showed similar neuroinvasion patterns as controls and those with V3000 showed similar MSTs as controls. Serum TNF-_ was increased in V3000-infected mice (peak - 52.9 pg/ml at 24 hours). However, mice given antibodies to TNF-_ had identical neuroinvasion patterns as controls.

These findings indicate that TM enhances neuroinvasion and pathogenicity in mice infected with VEE viruses but fail to indicate that pyridostigmine, LPS and TNF-_ affect VEE similarly, suggesting TM is unique in its effect on VEE virus. Tunicamycin could be a useful tool in further studies of VEE virus neuroinvasion.

**TUNICAMYCIN ENHANCES NEUROINVASION AND PATHOGENICITY IN MICE WITH VENEZUELAN EQUINE ENCEPHALITIS VIRUS**

By

Keith E. Steele

Dissertation submitted to the Faculty of the Department of Pathology of the Uniformed Services University of the Health Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, 2003

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| ANOVA | analysis of variance |
| ARGT | annual ryegrass staggers |
| BBB | blood brain barrier |
| BHK | baby hamster kidney |
| BSL-3 | biosafety-level 3 |
| CB | citrate buffer |
| CPE | cytopathic effect |
| EEE | eastern equine encephalitis |
| EMCV | encephalomyocarditis virus |
| ELISA | enzyme-linked immunosorbent assay |
| GAPDH | glyceraldehyde-3-phophate dehydrogenase |
| GFAP | glial fibrillary acidic protein |
| HE | hematoxylin and eosin |
| IC | intracerebral |
| IFA | immunofluorescent antibody |
| IP | intraperitoneal |
| IRF | interferon regulatory factors |
| LD | lethal dose |
| LPS | lipopolysaccharide |
| MOI | multiplicity of infection |
| MST | mean survival time |
| NBF | neutral-buffered formalin |

| | |
|---|---|
| NO | nitric oxide |
| PA | plaque assay |
| PB | pyridostigmine bromide |
| PBS | phosphate-buffered saline |
| PI | post inoculation |
| PFU | plaque-forming units |
| RPA | RNase protection assay |
| RT | room temperature |
| SEM | standard error of the mean |
| SFV | Semliki Forest virus |
| SV | Sindbis virus |
| TNF-_ | tumor necrosis factor alpha |
| TRD | Trinidad donkey |
| TUNEL | terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling |
| TM | tunicamycin |
| VEE | Venezuelan equine encephalitis |
| WEE | western equine encephalitis |

# I. INTRODUCTION

## A. Overview of Venezuelan Equine Encephalitis

Venezuelan equine encephalitis (VEE) virus causes explosive disease outbreaks in horses and humans throughout much of the tropical Americas. The virus has been weaponized, is highly infectious in aerosols[3,28] and is capable of producing prostrating illness or death among infected individuals. These characteristics make VEE virus a great concern as an agent of biowarfare and bioterrorism. Although a new vaccine candidate is in development,[1, 2] an approved, highly efficacious vaccine for VEE does not currently exist. Therefore, it is important to continue studies toward understanding the pathogenesis of VEE in order to provide avenues for developing prophylactic or therapeutic measures.

### 1. The Alphaviruses

VEE virus is a member of the genus alphavirus in the family Togaviridae. This group of single-stranded, positive-sense RNA viruses represents a variety of mosquito-transmitted diseases of medical and veterinary significance with overall global distribution and relatively diverse clinical syndromes.[3] Alphaviruses are maintained in nature by transmission cycles between mosquitoes and their vertebrate hosts, usually rodents and birds or in some cases nonhuman primates.

In humans, alphaviruses cause a number of often nonspecific, usually febrile illnesses.[3] In particular, however, encephalitis and arthritis are consistent and medically important manifestations of infection by several alphaviruses. These predominant clinical outcomes often correlate with the geographical

location of particular viruses. Thus, members of the genus are often categorized as those that manifest primarily as arthritis (old world viruses) or those that manifest primarily as encephalitis (new world viruses). In addition to causing natural disease, alphaviruses are also considered important experimentally for the study of general mechanisms of CNS diseases such as neuronal death (Sindbis virus) and CNS demyelination (Semliki Forest virus).[49, 54, 90]

In the Americas, there are 3 alphaviruses of particular importance because of their propensity to cause encephalitis in both horses and humans.[3] These include VEE virus, Eastern equine encephalitis (EEE) virus and Western equine encephalitis (WEE) virus. EEE virus is found in the eastern ranges from Canada to South America while WEE virus is found in the western portions of the Americas from Canada to Argentina. VEE virus primarily is found in northern South America and throughout Central America. During outbreaks, VEE virus may produce thousands of human infections, but the percentage of encephalitis cases that results in usually low.[7,8] In contrast, both WEE and especially EEE virus infections more commonly result in encephalitis, but infections due to these viruses are infrequent and sporadic.

## 2. VEE Virus Strains in Nature

VEE virus was originally identified in 1939 as the cause of equine disease in Venezuela.[4] Since then, a number of serologically closely related virus strains have been recognized and together these viruses form the VEE complex. There are currently 6 recognized subtypes in the VEE complex with additional variants within some subtypes.[3] These viruses are further grouped as epizootic and

enzootic strains. Epizootic VEE strains include subtypes IA/B and IC. IA/B and IC strains are pathogenic for horses, cause high viremia in them and are responsible for the periodic outbreaks. The remaining strains are not pathogenic for equines but cycle enzootically between rodents and mosquitoes. However, both epizootic and enzootic strains can cause serious disease in humans.[3] The epizootic and enzootic viruses are further distinguished by differences in plaque size, sensitivity to interferon, and virulence for guinea pigs. The epizootic VEE strains are also generally more virulent for laboratory rodents and nonhuman primates.

### 3. Transmission and Epidemiology of VEE Virus

Transmission of VEE viruses is by competent species of mosquitoes. Epizootic VEE strains can be transmitted by several genera, including several species of Aedes mosquitoes.[3] Enzootic strains are transmitted by Culex mosquitoes. Nonimmune equines become infected by mosquitoes carrying epizootic strains and then serve to amplify the virus. Infections in humans occur as the result of spillover from equine infections. Because horses do not amplify enzootic VEE viruses, most natural human illnesses and deaths are due to IA/B and IC viruses rather than the endemic strains.

Epizootics and accompanying epidemics of VEE are usually seen during periods of heavy rainfall when ample susceptible horses are available and an epizootic virus is circulating. Major outbreaks of VEE have primarily occurred in the northern countries of South America, but have arisen as far north as Mexico and have even extended into Texas. Enzootic strains of VEE continue to circulate

in south Florida, causing occasional cases of human disease.[5, 6] In a recent epidemic in Venezuela and Columbia, an estimated 75,000 to 100,000 people were infected, resulting in 3,000 neurologic cases and 300 fatalities.[7, 8] It was also estimated that as many as 8% of the horses in the affected areas died during this outbreak.

## 4. Clinical and Pathological Characteristics of VEE in Humans and Animals

VEE virus in humans produces a clinical syndrome manifest by fever, severe headache, myalgia, chills and occasional pharyngitis, lasting from a few days to two weeks.[7, 8, 9, 10] The incubation period is about 1 to 4 days.[10] The central nervous system is affected in a minority of cases, usually involving individuals under 17 years of age. Clinical manifestations of CNS disease in VEE may include convulsions and paralysis. Abortions and increased fetal deaths have also been attributed to VEE virus infection.[7, 8] Studies of chronic illness due to VEE virus are limited, however one report documented motor disorders and an increased incidence of seizures in children following VEE outbreaks.[11]

In fatal human cases of VEE, systemic pathologic changes include widespread congestion, edema and hemorrhage, lymphoid necrosis, hepatocellular degeneration, and interstitial pneumonia.[12] CNS changes include meningoencephalitis and myelitis characterized by infiltrates of lymphocytes, mononuclear cells and neutrophils, along with edema, congestion and hemorrhage and occasionally vasculitis. Multiple regions of the brain are affected.

A variety of animals models feature important aspects of the human disease[12] and have been used to study VEE. These include mice, rats, hamsters, guinea pigs, rabbits, monkeys, horses and burros.[3, 13, 14, 15, 16] Mice have been the most extensively used animal model. They exhibit a biphasic illness and develop both extraneural and CNS infection, like their human counterparts who develop encephalitis. Many aspects of the pathogenesis of VEE have been studied in the mouse, including experiments aimed at understanding the course of natural infection following vector transmission and the pathogenesis of aerosol VEE infection. In addition, mice have been used to study the immunologic and apparent immunopathologic response to VEE virus infection as well as a number of virulence factors. Other aspects of VEE in mice will be discussed in subsequent sections.

**5. Virology of Alphaviruses**

The structure and replication of alphaviruses has been studied in considerable detail. Most of the information has been gained by studying Sindbis virus, the prototype alphavirus, as well as Semliki Forest virus. Studies of other alphaviruses, including VEE virus, have also added to the general knowledge of the alphaviruses.

Alphaviruses have a relatively simple structure (Schlesinger,[17] Strauss,[18] and references therein). Virions exhibit icosahedral symmetry and have a diameter of about 70 nm. The viral genome is carried on a single strand of RNA, enclosed within a capsid that is surrounded by a lipid bilayer from the host cell membrane in which are embedded viral proteins. The alphavirus genomic RNA

is positive sense and measures about 12 kb in length. The capsid is composed of 240 copies of a single protein in an icosahedral formation. The lipid envelope contains 2 viral-encoded glycoproteins, E1 and E2. These form a glycoprotein coat made up of 80 trimers, each one of which is composed of 3 heterodimers of E1 and E2.

The VEE genome contains 11,444 nucleotides.[18] The 5' end of the genome codes for 4 nonstructural proteins (nsP1-nsP4), while the 3' end encodes the capsid protein, E1 and E2. Genomic RNA serves as the template for translation of the nonstructural proteins, but not for the structural proteins. A negative sense strand is transcribed from genomic RNA and serves two purposes. It is the template for generation of new genomic RNA as well as a 26S subgenomic positive sense fragment which itself serves as the template for synthesis of the structural proteins. Recombinant chimeric VEE viruses have shown that viral components in both the structural and nonstructural regions of the genome contribute to the ability to cause epidemics.[19]

The nonstructural proteins are formed as one or two polyproteins which are cleaved into nsP1, nsP2, nsP3, and nsP4, plus a number of functionally active cleavage intermediates. The structural proteins are produced as a single polyprotein from the subgenomic RNA. The polyprotein contains four regions, C (capsid), PE2 (also known as P62), 6K and E1. The C region, which is N-terminal in the polyprotein, contains serine protease activity and acts to cleave itself from the polypeptide as it is being formed. The capsid protein binds specifically to the new genomic RNA and induces nucleocapsid formation. Once

the capsid protein has been cleaved, the remainder of the polyprotein enters the endoplasmic reticulum, where further proteolysis occurs. The pE2, E1 and 6K proteins are reported to move together through the Golgi apparatus to the plasma membrane. pE2 forms dimers with E1 during the transit phase. The pE2 protein then undergoes additional cleavage by a host cell furin protease to form the mature E2-E1 heterodimer. The cleavage of E2 from PE2 results in the formation of the peptide E3, which is not incorporated into virions. This cleavage step is considered critical to virus maturation. It is required for the production of virions in insect cells and for the infectivity of the virus in vertebrate cells. Cleavage of the E3 protein from PE2 appears necessary to ensure the proper conformation of E2 in order for it to serve its function as the ligand by which alphaviruses bind to cell receptors in their mammalian hosts. This requirement also serves as the basis for vaccine development. An attenutated VEE virus strain (V3526) in which cleavage of E3 from PE2 is prevented by deletion of the furin-sensitive cleavage sequence is the vaccine candidate currently under development.[2]

**6. Infectious Alphavirus Clones**

A significant advancement in the study of alphaviruses was the development of full-length cDNA clones corresponding to the entire virus genome.[18] These constructs involve the placement of the viral genome downstream of a promoter for an RNA polymerase. Transfection of such clones into susceptible cells results in virus replication and the formation of infectious virions. An infectious cDNA clone of VEE virus, named V3000, was prepared from the virulent Trinidad donkey strain.[20, 21] The Trinidad donkey and V3000

strains have very similar *in vitro* and *in vivo* characteristics. cDNA clones offer several advantages for the study of alphavirus pathogenesis. Because RNA viruses have relatively high mutation rates, cDNA clones provide a stable, homogeneous source of infectious virus whose genetic sequence can be determined through DNA sequencing of the clone. Second, genetic analysis of different virus strains can be used to determine sites in the genome responsible for a variety of phenotypic characteristics, such as *in vitro* growth characteristics or *in vivo* pathogenetic features.

Site-directed mutants can be produced from the cDNA constructs and the effects of even single nucleotide changes on particular features of the virus can be determined. Such studies using the cDNA VEE virus clones have contributed considerable information to understanding of the pathogenesis of VEE.[22, 23, 24, 25, 26, 27, 28] Some important features of several VEE viruses used in studies of VEE pathogenesis are summarized in Table 1.

Table 1

## 7. Immunology of VEE Virus Infections

*In vitro* studies and *in vivo* studies with mice have been used to investigate the immune response to VEE virus. The development of serum neutralizing antibodies (IgG) appears necessary for protection against a peripheral infection with virulent VEE virus, as well as for other alphaviruses.[3, 29] Virus neutralization requires high avidity antibodies directed at specific epitopes on the E1 or E2 glycoproteins.[33, 34] Cellular immunity has not been shown to be protective against natural infection. Although serum IgG antibody protects against peripheral

**Table 1. Features of important VEE virus strains used experimentally.**

| Virus Strain | Genetic Features | Viremia[1] | Neuro-invasion[2] | Mortality in Mice | | |
|---|---|---|---|---|---|---|
| | | | | Peripheral Challenge | IC Challenge | Aerosol Challenge |
| TrD[3] | Wild-type | 100%/$10^6$ pfu/ml | 100% | 100% | 100% | 100 |
| V3000 | Derived from cDNA copy of TrD | 100%/3 x $10^5$ pfu/ml | 100% | 100% | 100% | 100 |
| TC-83 | Live-attenuated derivative of TrD | 60%/200 pfu/ml | 0% | 0% | 0/100%[4] | 0/100%[4] |
| V3526 | Cleavage mutant of V3000[5] | 100%/200 pfu/ml | 0% | 0% | 0% | 0% |
| V3010 | E2 76 (Lys) | 100%/3 x $10^5$ pfu/ml | 30% | 0% | 22% | ND[6] |
| V3014 | E1 272 (Thr)<br>E2 209 (Lys)<br>E2 239 (Asn) | 0% | 0% | 0% | 100% | ND |
| V3032 | E2 209 (Lys) | 50%/6 x $10^3$ pfu/ml | 10% | 0% | 89% | ND |
| V3034 | E1 272 (Thr) | 100%/$10^5$ pfu/ml | 40% | 11% | 11% | ND |

[1] Viremia is defined both as the percentage of infected mice that develop detectable serum titers and as the average peak serum titer.
[2] Neuroinvasion is defined as the percentage of mice in which virus can be detected in the brain after peripheral (footpad or subcutaneous) infection from 48 hours pi onward. [3] TrD is the Trinidad Donkey isolate. [4] Mortality with TC-83 is 0% in infected Balb/C mice but 100% in infected C3H/HeN mice. [5] The entire 4 amino acid furin cleavage sequence encoding the glycoprotein PE2 is deleted from the V3000 parent strain and combined with a second mutation in E1 253 (Ser). The furin cleavage sequence is normally required for cleavage of the 55 AA glycoprotein E3 from PE2; therefore, E3 remains as part of PE2 in the V3526 virion. [6] Not Determined. Information extracted from previous reports.[2, 28, 29, 30, 31, 32]

challenge, neutralizing IgA antibody may be required to protect mice against aerosol challenge with VEE virus.[2, 29]

More recent studies have identified additional immunological mechanisms involved in the host response to VEE virus infection[35, 36, 37] Infection of mice with virulent VEE virus results in upregulation of a number of proinflammatory mediators in the brain, including IFN-α, IFN-β, IL-1α, IL-1β, IL-6, IL-10, IL-12, TNF-α, and iNOS.[35, 38] Various interleukins, including IL-1β, IL-2, IL-4 and IL-6 have also been shown to be up-regulated in the CNS of mice infected with Semliki Forest virus (SFV).[39] IFN-α/β appears to play an important early protective role in VEE infections[35, 36, 37] IFN-α/β-receptor knockout mice are more susceptible to infection both with virulent and attenuated VEE virus strains. These mice show increased dissemination of virus to tissues, greater destruction of lymphoid tissue, and significantly decreased survival times. Normal mice likewise exhibit more severe disease from virulent VEE virus following treatment with antibodies to IFN-α/β. Conversely, treatment of mice with a novel formulation of IFN-α has been reported to protect mice against either a peripheral or aerosol exposure.[40] Mice with knockouts in interferon regulatory factors (IRF-1 and IRF-2) also exhibit greater susceptibility to infection with VEE virus. Interferon has also been shown to be protective against SFV infection.[41] In contrast to the protective effects of IFN-α/β, TNF-α and iNOS appear to contribute to the pathogenesis of VEE, as TNF-α and iNOS knockout mice have

extended survival times following VEE infection, although the overall mortality rates do not differ from normal mice.[38]

## B. Pathogenesis of VEE

### 1. Virus Kinetics Following Peripheral and Aerosol Infection

To model the natural transmission of VEE virus by mosquito bite, mice are experimentally infected by subcutaneous or footpad inoculation. A number of key steps in the pathogenesis of VEE have been elucidated in recent years using the mouse model. It has been shown, for example, that following footpad innoculation, dendritic cells in the dermis are the first type of cell to be infected.[27] Infected dendritic cells appear to transport virus to the draining lymph node, where initial replication occurs. Virus can be detected in the lymph node by 4 hours post inoculation (PI) and virus titers of $10^6$ to $10^7$ plaque-forming units (PFU) per gram of tissue are present in the draining lymph nodes by 6 hours pi.[24, 26] Infection of macrophages may also be important in the early pathogenesis of VEE. Early infection of mononuclear phagocytes in lymph nodes by V3000 has previously been shown. [42] It has also been demonstrated that the virulence of a VEE virus strain correlates with its ability to replicate rapidly in macrophages.[31]

By 12 to 24 hours PI, VEE virus is already present in the blood and in other lymphoid tissues such as the spleen, non-draining lymph nodes and thymus.[26] Virus seeding of nonlymphoid tissues including heart, lung, liver, pancreas, kidney, adrenal gland and salivary gland also occurs at this time. Clearance of virus from the blood and peripheral tissues occurs relatively rapidly,

being essentially complete by about 3-4 days PI. However, invasion of the brain occurs prior to peripheral clearance. Virus can be detected in the brains of mice by 48 hours PI in mice infected by the footpad or subcutaneous routes.[26, 42, 43]

The E2 glycoprotein appears to play an important role in the targeting of dendritic cells by VEE virus. A V3000 mutant, designated V3010, created by point mutation at E2 76 (Glu-Lys), failed to infect dendritic cells in vivo, did not spread beyond the lymph node draining the site of inoculation, and was avirulent in mice.[27] A strain produced by insertion of a second site reversion mutation at E2 116 (Glu-Lys) into the V3010 strain, designated V3533, had restored ability to infect dendritic cells, the ability to spread to lymphoid tissues and partially restored virulence.

Additional VEE mutations have been shown to correlate with other steps in the spread of VEE virus through the body following peripheral infection.[26] V3014, which differs from V3000 at three loci (E2 Lys 209, E2 Asn 239, E1 Thr 272) spreads to the draining lymph node but is blocked from further spread. V3032 (E2 Lys 209) spreads to the draining lymph node and other lymphoid tissues but it does not produce significant viremia or CNS invasion. V3034 (E1 Thr 272) produces near normal replication in peripheral tissues, but only sporadically infects the CNS. Several of the V3000 mutants used to infect mice have been shown to develop reversion mutations that allow them to overcome the initial block to their spread in the body, but nonetheless do not permit them to regain full virulence.[22] The tropism of a variety of VEE virus stains is summarized in Figure 1.

Fig. 1

VEE virus invasion of the brain is a critical event in the pathogenesis of VEE and has been the subject of several studies.[28, 30, 31, 42, 43, 44, 45] Animals infected with VEE virus by aerosol administration exhibit early massive infection of the olfactory mucosa and virus makes its first appearance in the brain in the olfactory bulbs. This is not surprising, as the so-called bipolar or olfactory sensory neurons in the olfactory neuroepithelium are in direct contact with the environment,[46] and as these cells are a strong and specific target of aerosolized virulent VEE virus. The opposite poles of olfactory neurons are axonal processes called fila olfactoria that coallesce to form the olfactory nerves. The olfactory nerves pass through the cribriform plate of the skull and axons synapse directly with neurons in the olfactory bulbs of the brain. Therefore, a single cell which contacts the environment has a direct connection to the brain. Infection of these olfactory neurons, then, appears to be the major route of CNS infection following aerosol administration of VEE virus.

When mice are inoculated subcutaneously or by footpad administration with fully virulent VEE virus, infection of the brain also appears first within the olfactory bulbs.[42, 43] Contrary to aerosol infections, however, the neurons in the olfactory neuroepithelium do not appear to be significantly infected prior to virus entry into the brain. Importantly, one study showed that VEE virus in the blood of mice seeded the perivascular areas in the connective tissue underlying the olfactory neuroepithelium within 18 hours of peripheral infection;[43] i.e. prior to the appearance of virus in the brain. The specific manner by which VEE virus exits the vasculature has not been demonstrated. Compared to aerosol infection, there is a delay in the appearance of virus in the brains of peripherally-infected

| | |
|---|---|
| **V3000** | Footpad → Dermal dendritic cells → Draining lymph node → Viremia → Fila Olfactoria → Brain |
| **V3010** | Footpad -\| |
| **V3010 revertant*** | Footpad → Dermal dendritic cells (presumed) → Draining lymph node → Viremia → Brain |
| **V3533** | Footpad → Dermal dendritic cells → Draining lymph node -\| |
| **V3014** | Footpad → Dermal dendritic cells (presumed) → Draining lymph node -\| |
| **V3032** | Footpad → Dermal dendritic cells (presumed) → Draining lymph node → Additional lymphoid tissues -\| |
| **V3034** | Footpad → Dermal dendritic cells (presumed) → Draining lymph node → Viremia -\| |
| **V3526** | Subcutaneous → → Viremia-\| |
| **TC-83** | Subcutaneous → → Viremia-\| |
| **TrD** | Aerosol → Olfactory neuroepithelium → Brain, lymphoid tissues, visceral tissues, teeth |
| **V3526** | Aerosol → Olfactory neuroepithelium → Brain, lymphoid tissues |
| **TC-83** | Aerosol → Olfactory neuroepithelium → Brain |

**Figure 1. Tropisms of VEE virus strains in mice**. Mice were infected experimentally with virulent (V3000 or Trinidad donkey (TrD)) or attenuated strains of VEE virus by the peripheral and aerosol routes.[22, 26, 27, 28, 30] The step where a strain is blocked from further spread is indicated by -| These virus strains are defined in Table 1.

* - The V3010 revertant develops a second mutation (E2 116, Glu to Lys) *in vivo* that permits progression to the brain but does not provide full virulence.

mice of about 12-24 hours, a lag period that pretty closely corresponds to the time necessary for the development of peak viremia.

An additional route of neuroinvasion appears to involve the teeth, which are also an early target of VEE virus[28, 42, 43] Infection of the dental pulp, which is innervated by sensory branches of the trigeminal nerve, appears to lead to infection of the brainstem in the region of the nucleus of the trigeminal nerve. This means of neuroinvasion appears to occur after infection of the olfactory bulbs, though, and therefore is likely not of primary significance. A third route that has been proposed is direct infection of the brain via the vasculature.[45] However, infection of endothelial cells appears limited, occurs relatively late in the course of disease, and there is no perivascular pattern of virus entry into the brains of infected animals to strongly support this notion.

### 3. VEE Virus Kinetics in the Brain

Within the brain, neurons are the major target of virulent VEE virus in the mouse.[16, 28, 38, 42, 43] Other encephalitic alphaviruses also target neurons. From the olfactory bulbs, VEE virus spreads first to structures in the brain that receive efferent connections from the olfactory bulbs and then to remaining regions of the brain in a generally rostral to caudal fashion. Rostral portions of the brain such as the pyriform cortex and thalamus become infected early, while more caudal structures like the brainstem and cerebellum generally are infected later. Infection then proceeds into the spinal cord, where anterior horn neurons are the major target. There is a noticeable bilateral symmetry to the pattern of antigen deposition, indicating that particular tracts are responsible for virus spread in the brain.[28] The pattern of virus distribution through the brain appears identical following infection by either the peripheral or aerosol routes.

In addition to neurons, cells in the brains of mice with morphological features of glial cells also appear to be infected, although not to the extent that neurons are. Wild-type VEE virus and TC-83, both of which spread readily in the brain, appear to primarily infect neurons and some glial cells. The molecularly-cloned strain V3526, which shows limited neurovirulence following aerosol administration, appears to infect only small neurons and/or glial cells.[28] Specific identification of the types of glial cells infected *in* vivo with VEE virus using double-labeling or other means has not been reported. VEE virus has been shown to infect astrocytes and microglial cells *in vitro*, however.[37, 38] In other animals, virulent VEE virus appears to infect neurons in the brain less well than in mice. Equines exhibit moderate neuronal infection, while monkeys apparently have limited infection of neurons.[16] Infection of glial cells in these species has not been reported.

**4. Neuronal Death**

The mechanism of neuronal cell death in alphavirus infections of the brain has been the subject of extensive investigation. It is believed that the increased susceptibility to VEE and other alphavirus encephalitides among children may be explained by the increased susceptibility of immature neurons to undergo apoptosis following infection.[47] A mouse model using the prototype alphavirus Sindbis virus (SV) has been used to investigate the roles of neuronal infection and apoptosis in the pathogenesis of alphavirus encephalitis. The outcome of SV encephalitis in the mouse appears to be significantly influenced by the strain of virus used and the age of the mice infected.[48] Some strains of SV cause fatal encephalitis in newborn mice, but weanling mice are resistant.[49] Older mice, however, are susceptible to a more neurovirulent strain.[49, 50] The greater susceptibility of newborn mice to certain strains correlates with widespread

apoptosis of neurons in the CNS. Infected neurons of older mice do not exhibit significant apoptosis with these less neurovirulent strains, but they do following infection with the more virulent strain.[50, 51]

Experimental mouse models of VEE virus infection have also been used to study the role of apoptosis in neuronal destruction. Separate studies have reported that apoptosis is the cause of neuronal cell death in mice infected with VEE virus.[38, 52] These studies have relied on the use of terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining for apoptosis and identification of neurons by morphology with light microscopy.

**5. Immunopathology and Neurodegeneration**

Inflammation of the brain is a significant component in the CNS of humans and animals infected with VEE virus. Mice, equines, monkeys and humans with VEE exhibit meningoencephalitis characterized by cellular infiltrates in the meninges and perivascular spaces.[12, 15, 16, 28] These infiltrates comprise predominantly lymphocytes, but are usually mixed with some histiocytic cells and neutrophils. Neutrophils are particularly prominent in equine cases, but are also present in the brains of affected mice and humans. Inflammatory cells often infiltrate the parenchyma in infected foci as well.

The possibility that immunopathologic mechanisms might contribute to the pathogenesis of VEE was suggested years ago when it was shown that treatment with antithymocyte serum prolonged the survival time of mice infected with VEE virus.[53] Further support for the notion that VEE involves immune-mediated disease was provided by a study that showed SCID mice with VEE lacked the cellular inflammatory changes in the brain that immunocompetent mice had, and survived about 3 days longer than immunocompetent mice.[3] In another

study, C3H/HeN mice primed with the TC-83 vaccine strain peripherally and exposed to virulent VEE virus by aerosol developed much more severe inflammatory infiltrates in the brain following infection than did unprimed mice.[2] Even though the TC-83-primed mice had less virus and less neuronal necrosis than did unprimed mice, nearly all of them died, on average just 1 day more than the unprimed mice.

Experiments have also implicated astrocytes for having a role in the neurodegeneration seen with VEE.[32, 38] One study reported that degeneration of neurons occurred in portions of the brain associated with astrogliosis in which viral infection was not apparent.[38] This study also reported that the brains of VEE-infected mice expressed increased levels of apoptosis-signalling molecules such as TNF-α, Fas ligand, TRAIL, TNFr p55, TNFr p75 and Fas antigen, although the cells expressing these mediators were not identified. In a separate study, VEE infection of primary astrocytes was shown to result in increased expression of the proinflammatory mediators TNF-α and iNOS.[32] These experiments suggest that astrocytes may contribute to the damage to neurons that occurs following VEE virus infection.

## C. Viral Neuroinvasion and Neurodegeneration

### 1. General Mechanisms of Viral Neuroinvasion

There are generally two major routes by which viruses gain entry into the brain. The first is by ascending transmission after primary infection of the peripheral nervous system. Infections of the brain by rabies virus and herpes viruses are classical examples of this form of neuroinvasion. In effect, aerosolized-VEE virus infection of the brain represents a specialized case of this because the virus ascends through the olfactory nerves. The second route by

which viruses infect the brain is the bloodstream. Usually, this involves the exit of virus directly from vessels in the brain. Examples of viruses that utilize a hematogenous route to directly infect the brain include HIV, Japanese encephalitis virus, HTLV-1 and Semliki Forest virus (SFV).[54, 55, 56, 57] Arboviruses in particular appear to use a variation of this means. Virus particles exit the bloodstream, but from vessels in the olfactory tract rather than the brain. Then they infect the olfactory neurons of the olfactory tract. From here, virus ascends into the olfactory bulbs via the olfactory nerves, followed by subsequent spread throughout the brain. St. Louis encephalitis virus,[58] yellow fever virus[59] and louping ill virus,[60] as well as VEE virus,[43] are examples of arboviruses that use this route.

Relatively little, however, is known about the specific mechanisms by which hematogenous viruses are able to exit the bloodstream, a process that is essential for invasion of the CNS. Most of these viruses, however, probably exit the vasculature by one of two ways. The first involves transcytosis, either passively within endocytotic vesicles or requiring active infection of the endothelial cells. The second is by the paracellular route; i.e. viruses pass between endothelial cells. HIV-1, JE virus, HTLV-1 and SFV have all been reported to involve endothelial cell transcytosis.[54, 55, 56, 57] HIV-1 has also been reported to utilize the paracellular route.[61] Transcytosis of SFV, HTLV-1 and HIV-1 have all been associated with upregulation of the adhesion molecule ICAM-1 on the surface of brain endothelial cells.[54, 55, 56] Transcytosis of SFV and HTLV-1 have also been associated with upregulation of VCAM-1. Similar studies investigating the process of virus egress from the vasculature of the olfactory tract appear lacking.

**2. Factors that Alter Viral Neuroinvasion**

Differences in the barriers to neuroinvasion that exist among individuals may help explain the apparent differences in susceptibility to viral encephalitides. A variety of factors that alter host barriers to neuroinvasion and that could conceivably increase the likelihood of developing viral encephalitis have been the subject of several studies in recent years. In particular, factors which are reported to alter the blood brain barrier (BBB) appear important. For example, external factors such as gram-negative bacteria or lipopolysaccharide (LPS) alone induce structural and functional changes in the BBB of mice challenged experimentally.[62, 63, 64] Further, it has been shown that LPS increases viral neuroinvasion and the severity of encephalitis caused by West Nile virus and Sindbis virus.[64] Also, host factors such as stress, ischemic disease and more specific determinants like tumor necrosis factor alpha (TNF-α) and nitric oxide (NO) have been implicated as important factors that may alter the BBB.[65, 66, 67, 68] TNF-α upregulation in the BBB has been associated with neuroinvasion by HTLV-1 and HIV-1.[55, 61] In addition, breakdown of the BBB that occurs during dengue virus infection has been attributed to the effects of a virus-induced, host cytokine called cytotoxic factor.[69]

Chemical agents are additional factors that have been shown to alter the BBB.[70, 71] Acetylcholinesterase inhibitors including pyridostigmine and others have previously been shown to alter the BBB,[72, 73] and to increase the ability of Sindbis virus to invade the CNS.[71] Also, cocaine has been associated with BBB alteration and with neuroinvasion by HIV-1.[61]

### 3. Altered Blood Brain Barrier and Neurodegeneration

In addition to facilitating viral entry into the brain, abnormalities in the BBB have been reported to mediate tissue damage in a variety of neurodegenerative conditions.[74, 75, 76, 77] The BBB abnormalities associated with tissue damage in the brain may vary from subtle [78] to severe and generally involve two types of pathogenetic events. BBB alterations may allow circulating leukocytes such as macrophages and lymphocytes to more easily emigrate from the bloodstream into the brain, where their damaging effects are manifest. Secondly, extravasation of serum proteins may induce direct changes in glial cells and neurons or may induce localized inflammatory changes.

## D. Tunicamycin

Tunicamycins are nucleotide antibiotics produced by several species of *Streptomyces*. The structure of tunicamycin (TM) consists of uracil, N-acetylglucosamine, an 11-carbon 2-aminodialdose sugar and an amide-linked fatty acid.[79] Structural variants of TM differ in their fatty acid constituents. Tunicamycin is a potent inhibitor of N-linked glycosylation of proteins and is commonly used in experimental cell biology. A group of toxins called corynetoxins are products of *Clavibacter toxicus* and are very closely related to TM in structure and biological activity. These toxins may contaminate *Lolium rigidum* (annual ryegrass) and are responsible for the animal disease known as annual ryegrass toxicity.

Our laboratory previously showed that TM caused enhanced mortality due to SFV and EMCV, which correlated with increased levels of these viruses in the

brain.[41] Preliminary data in our laboratory also suggested that TM might increase the pathogenicity of VEE virus as well.

**1. Possible Mechanisms of TM-enhancement of Viral Encephalitis**

Tunicamycin has two important characteristics that could be involved in enhancing encephalitis due to VEE virus or other encephalitic viruses. First, like other agents shown to enhance viral neuroinvasion, TM has been shown to alter the BBB.[70] In this study, guinea pigs treated with 400 μg/kg of TM demonstrated loss of the integrity of the BBB to the tracer molecule horseradish peroxidase. This damage was relatively subtle, however, as the guinea pigs retained barrier function to the larger molecules ferritin and colloidal gold.

Secondly, because of its ability to inhibit N-linked glycosylation of proteins, TM has the potential to alter viral pathogenesis through a number of means. Generally these could involve altered viral glycoproteins (E1 and E2) or a variety of host glycoproteins. In particular, host glycoproteins such as those of the immune system, including interferons and a number of interleukins, as well as a number of adhesion molecules, are important potential targets of TM. Also potentially important are host cells glycoproteins that may be involved in virus trafficking.

**E. <u>Synopsis</u>**

VEE virus will likely remain a threat to civilian and military populations through continued epidemics and the possible use of the virus as an agent of biowarfare or bioterrorism. Although many advances have been made in recent years, much remains to be understood about the specific viral and host

mechanisms that influence the pathogenesis of VEE and factors that protect a susceptible host from disease.

Future studies are likely to continue to focus on the host mechanisms that operate to prevent or control VEE virus infection and therefore limit the progression of disease. Such studies should investigate the host and viral mechanisms that are responsible for the development of encephalitis. In particular, the viral and host factors that contribute to neuroinvasion and neurodegeneration require further investigation.

## F. Specific aims, significance, hypothesis and approach

The primary aim of this research is to determine whether chemical agents, in particular TM, alter the pathogenesis of VEE in mice. Secondarily, demonstrating the means by which TM or other chemicals affect VEE is an important objective. The major specific aims of this research include the following:

1. Determine whether TM alters the development of viremia, neuroinvasion and replication of virus in the brains of mice infected with virulent and attenuated VEE viruses.
2. Characterize the effect of TM on the neurodegenerative changes, clinical disease and mortality in mice infected with virulent VEE virus and on the integrity of the BBB in uninfected mice.
3. Determine whether LPS, pyridostigmine and TNF-_ enhance VEE in mice.

Overall, it is hoped that achieving these specific aims will contribute to a better understanding of the mechanisms by which VEE virus invades the central nervous system and perhaps also the means by which neurodegenerative changes develop.

Determining if and how TM influences VEE could provide the basis for using this chemical as an experimental tool to study the pathogenesis of this disease. Ultimately, a better understanding of how VEE virus invades the central nervous system and how neurodegenerative changes in the brain develop as the result of CNS infection could lead to ways of preventing or limiting the damaging effects of VEE. This research is also biologically relevant, because humans and animals could be exposed to TM or other chemicals naturally or by their deliberate use and thus become more susceptible to VEE or other viral encephalitides. Demonstrating host factors that play roles in VEE would also contribute to the general understanding of this disease.

The hypothesis for this research is that TM, and possibly other external or host factors enhance the pathogenicity of VEE. More specifically, TM-induced enhancement of VEE is mainly due to enhanced neuroinvasion mediated by its adverse effect on the BBB. This hypothesis is supported by previous studies showing that TM and a variety of other factors known to damage the BBB have also been associated with neuroinvasion by several viruses. It is further supported by previously reported and recent studies in our lab showing that TM enhances encephalitis due to SFV, encephalomyocarditis virus (EMCV) and possibly VEE and that pyridostigmine also enhances viral encephalitis due to SFV and EMCV.

The approach to meeting the aims of this research will employ a combination of biological, morphological, virological and molecular methods with the mouse model of VEE that has been extensively characterized in this laboratory and others. Specifically, virus plaque assay and

immunohistochemistry of brain histologic sections will be applied to measure the processes of neuroinvasion and virus replication and spread through the brain. The process of neurodegeneration will be measured using histologic and immunohistochemical techniques to demonstrate inflammation, neuronal damage and gliosis. These will be supplemented by methods that can demonstrate the expression of factors in the brain indicative of neurodegeneration, such as the RNase protection assay (RPA) for cytokine genes and other host factors. Potentially important serum factors like TNF-□ and NO will be measured by standard protein assays such as ELISA and the Griess reaction. Alteration of the BBB will be examined using electron microscopy and/or immunohistochemistry for the demonstration of leakage of blood proteins.

These methods will be applied to assess the pathogenesis of VEE during the pre-neuroinvasion, neuroinvasion and neurodegeneration phases. Appropriate comparisons between experimentally-manipulated and control mice will be made. The statistical significance of data will be determined by standard statistical methods as suitable.

It should be noted that this approach is designed to provide scientifically valid data while ensuring that necessary safety concerns are met. VEE virus is a biosafety-level 3 (BSL-3) agent that requires that live virus be used only within the BSL-3 containment laboratory and that any biological samples removed for analysis outside the BSL-3 laboratory must ensure that virus is completely inactivated. These factors may limit the experimental methods available for use in the current studies.

## II. MATERIALS AND METHODS

### A. Mice

Six to 10-week-old CD-1 mice (Charles River Laboratories, Wilmington, MA) were obtained and used for all in vivo experiments. Female mice were used for the V3000-tunicamycin (TM) mortality experiment and the V3000-pyridostigmine bromide (PB) mortality experiment 1. Male mice were used for all remaining studies. Mice were housed in cages equipped with microisolators and were provided food and water ad libitum, except as noted. For the portions of the study involving live VEE virus, mice were housed in a biosafety level 3 (BSL-3) facility accredited by the American Association for Accreditation of Laboratory Animal Care. A 12 hour light/dark cycle was maintained in the animal room. In conducting research with mice, the investigators adhered to the *Guide for the Care and Use of Laboratory Animals* (Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council, NIH Publication No. 86-23, revised 1996).

### B. Viruses and chemical agents

For experiments with VEE viruses, the molecularly-cloned, virulent strain V3000 and the attenuated strains V3010 and V3034, as previously reported,[21, 26] were used. Preparations of VEE virus stocks and virus titrations were also performed as reported. Briefly, virus working solutions were prepared by dilution of virus stocks in 1X PBS containing Ca++ and Mg++ supplemented with 0.1% bovine serum. All experiments with live VEE viruses were conducted in the BSL-3 laboratory. Semliki Forest virus (SFV) was purchased from the American

Type Culture Collection (ATCC, Manassas, VA) and encephalomyocarditis virus (EMCV) was obtained from C. Buckler (NIAD, NIH, Bethesda, MD). SFV and EMCV were passaged by intracerebral (IC) inoculation into CD-1 mice. Mice showing signs of encephalitis were sacrificed, the brains removed and 10% (w/v) suspensions were made in phosphate-buffered saline (PBS). Virus titers were determined using cytopathic effect (CPE) assays with BHK-21 cells (ATCC) for SFV and $L_B$ cells (B. Durke, University of Warwick, Coventry, UK) for EMCV.

Stock solutions of TM (Calbiochem, San Diego, CA) were prepared by dissolution in dimethylsulfoxide and working solutions were prepared by dissolution in sterile PBS. Pyridostigmine bromide (PB -Sigma) stock and working solutions were prepared by dissolution in propylene glycol. Lipopolysaccharide (LPS - Sigma-Aldrich; 0111:B4, Cat. # L2630) working solutions were prepared by dissolution in sterile, pyrogen-free PBS.

**C. Mortality studies**

Mice in individual studies were treated as described below. For all mortality studies, mice were observed daily and the numbers of dead mice were counted at 24-hour intervals for 14 days. The percentage mortality and mean survival time ± standard error of the mean (MST± SEM) for each group of mice were calculated.

**1. V3000-Tunicamycin**

For this study, mice were treated by intraperitoneal (IP) injection of 250 □l of diluted TM (5 □g/mouse) 6-12 hours before infection. Control mice were similarly treated with diluent alone. Mice were lightly anesthetized with

methoxyflurane and infected with 100 pfu (lethal dose) of the virulent VEE virus V3000 by inoculation of 25 µl of diluted virus stock into the left rear footpad.

**2. V3000-Pyridostigmine Bromide**

Mice were fasted 12 hours or overnight prior to PB administration. PB-treated mice were given 200 µl of working solution (1.09 mg/kg) by gavage, as follows: In V3000 experiment 1, mice were treated 24 hours before infection. In V3000 experiment 2, mice were treated 30 minutes before infection. In V3000 experiment 3, mice were treated 24 hours after infection (to coincide with expected peak viremia for V3000). A few mice died within several hours of PB administration and were excluded from the experiments. PB-treated and diluent-treated control mice were lightly anesthetized with methoxyflurane and inoculated with 100-1000 pfu of V3000 in volumes of 25 µl in the left rear footpad.

**3. V3010-Pyridostigmine Bromide**

Mice were anesthetized and inoculated with 1000 pfu of V3010 in the footpad, then were treated with PB or diluent (as described previously) 36 hours after infection (to coincide with expected peak viremia for V3010). Because V3010 immunizes mice against with virulent VEE virus, all mice that survived V3010 infection in this experiment were challenged to ensure initial infection. To accomplish this, 21 days after the initial infection with V3010, mice were anesthetized and inoculated with $1x10^4$ pfu of V3000 intraperitoneally. Mice were then observed daily for 14 more days. All of these mice survived V3000 challenge.

The V3010 strain was chosen for this experiment because it produces viremia and causes greater mortality after IC inoculation (20%) than after footpad inoculation (0%),[26] indicating that an increased amount of virus in the brain correlates with greater mortality. V3034 virus also produces viremia, but has the same mortality after IC inoculation (11%) as after footpad inoculation (11%), indicating that an increased amount of virus in the brain does not correlate with greater mortality. This was considered important because it was believed that if PB increased mortality from either attenuated strain, it would likely be due to enhanced neuroinvasion. The other available attenuated strains, V3014 and V3032, were not considered suitable because they produce little or no viremia, which is required for neuroinvasion.

**4. V3000-Lipopolysaccharide**

LPS-treated mice were given 200 μl of working solution (100 μg/mouse) by IP injection at the time of infection. LPS-treated and diluent-treated control mice were lightly anesthetized and inoculated with 1000 pfu of V3000 in 25 μl in the left rear footpad.

**5. Semliki Forest Virus-Pyridostigmine Bromide**

Mice were treated with 1.09 mg/kg of PB, as described. Twenty-four hours later, mice were infected with a sublethal dose (1 $LD_{50}$) of SFV by subcutaneous inoculation in a volume of 0.25 ml.

**6. Encephalomyocarditis Virus-Pyridostigmine Bromide**

Mice were treated with 0.56 mg/kg of PB, as described. Twenty-four hours later, mice were infected with a sublethal dose (1 $LD_{50}$) of EMCV by

subcutaneous inoculation in a volume of 0.25 ml.

**D. VEE-Tunicamycin Pathogenesis Studies**

A series of experiments were performed to investigate mechanisms by which TM might alter the pathogenesis of VEE in mice.

**1. V3000 Pathogenesis Following Footpad Infection**

The first pathogenesis experiment measured the effects of TM administration on key steps in the course of VEE in mice inoculated with the virulent strain. Mice were administered 2.5 μg TM (TM+) or diluent (TM-). This sublethal dosage was chosen based on a previous experiment that indicated the lethal dose of TM in adult mice to be 10 μg per mouse (Maheshwari *et al*, 1983). In that study, mice treated with TM at 5.0 μg or less exhibited no abnormal physical or pathological changes. Twelve hours after TM treatment, TM+ and TM- mice were lightly anesthetized with methoxyflurane and infected with 1000 pfu of V3000 (VEE+) in the footpad. Uninfected control (VEE-) mice were administered diluent in the footpad. All mice were observed daily for clinical signs of disease. Mice were killed at 6, 12, 24, 48, 72, 96, and 120 hours post infection (PI) to include the following experimental groups: VEE+/TM+ (N=4-7), VEE+/TM- (N=4-7), VEE-/TM+ (N=2), and VEE-/TM- (N=2). At each time point, mice were deeply anesthetized with methoxyflurane and killed by cervical dislocation. Mice were weighed (except the 12 hour group) and necropsies were performed immediately. The axillary vessels were transected and samples of blood were obtained. Each brain was removed and hemi-sectioned. One half of each brain was fixed in 10% neutral-buffered formalin and the remaining half was

processed for virus plaque assay (PA) and RNA analysis. Fixed brains were processed for histopathology and immunohistochemistry.

**2. V3000 Replication in the Brain Following Intracranial Infection**

The second pathogenesis experiment was designed to determine the effect of TM on the replication of virulent VEE virus in the brain. For this experiment, mice were treated with 2.5 μg of TM or diluent 24 hours before IC infection. This timing was chosen to approximate the effect of TM at the time when VEE virus likely began to replicate in the brain in the first pathogenesis experiment, expected to be approximately 24 hours after cutaneous inoculation based on a previous study.[43] TM-treated and diluent-control mice were anesthetized and infected with 25 μl containing 100 pfu of diluted V3000 virus injected into the left cerebral hemisphere. Three VEE+/TM+ and 3 VEE+/TM- mice were deeply anesthetized and killed at 2, 12, 24, 48, and 72 hours PI. Immediately after death, the right half of each brain was removed and processed for virus titration.

**3. Viremia and Neuroinvasion with V3034**

The third pathogenesis experiment investigated the effect of TM on neuroinvasion by the attenuated VEE virus V3034. Groups of mice were treated with TM or diluent as in the first pathogenesis experiment, anesthetized and infected with 25 μl containing 100 pfu of V3034 in the footpad. TM-treated and untreated mice were anesthetized and killed, 4 to 8 per group, at 48, 72 and 96 hours PI. Serum and brain samples were harvested at necropsy for virus titration as previously described.

## E. The Effect of Pyridostigmine and Lipopolysaccharide on Neuroinvasion by V3034

These experiments were performed to determine if PB and LPS, 2 agents reported to enhance viral neuroinvasion, had any effect on neuroinvasion by VEE virus. V3034 was chosen for these experiments because the V3034-TM experiment already described provided data with which to compare that obtained from these studies.

### 1. Pyridostigmine Bromide-V3034 Neuroinvasion

Mice were lightly anesthetized and infected with 1000 pfu of V3034 in the footpad. Mice were then fasted overnight. Twenty hours PI (approximate time of expected peak viremia for V3034), mice were treated with 1.09 mg/kg of PB or diluent, as described previously. Mice were deeply anesthetized and killed, 4 per group, at 72, 96 and 120 hours PI. The brains of all mice were removed and processed for virus titration.

### 2. Lipopolysaccharide-V3034 Neuroinvasion

For this study, 2 separate experiments were done. In experiment 1, mice were treated with 100 _g of LPS by IP injection, then were lightly anesthetized and inoculated with 1000 pfu of V3034 in the footpad. Mice were killed, 3 per group, at 48 and 72 hours PI. The brains were removed and processed for virus titration. These samples were taken in the course of experiment F.1, further described subsequently. The data regarding the brain virus titers were analyzed for inclusion here in retrospect. Because data were available only for 48 and 72 hours PI, however, a second experiment was performed. In experiment 2, mice were manipulated as for experiment 1, except that the LPS treatment was

performed at 22 hours PI (approximate time of expected peak viremia for V3034). Mice were anesthetized and killed at 48, 72 and 96 hours PI and the brains of mice were removed and processed for virus titration.

## F. Possible role of TNF-α and Nitric Oxide in VEE Virus Neuroinvasion

Because increased levels of TNF-α and nitric oxide (NO) have been associated with damage to the blood-brain barrier, the possible role of these 2 molecules in neuroinvasion by VEE virus was explored. This was accomplished in 2 related experiments.

### 1. Serum Levels of TNF-α and Nitric Oxide During Early VEE Virus Infection.

To determine if TNF-α and nitric oxide levels were increased during the early stages of VEE virus infection, i.e. at or prior to neuroinvasion, sera from mice infected with V3000 and V3034 were harvested and TNF-α and total nitrite levels were determined. Mice were anesthetized as previously described and were inoculated with 1000 pfu of V3000 or V3034 in the footpad, also as described. At 2, 12, 24, 48 and 72 hours PI, 3 mice per experimental group were deeply anesthetized, killed by cervical dislocation and serum and brain samples were harvested. The brain samples were processed for virus titers, as explained in experiment E.2. Serum samples were stored at -85°C and later analyzed for TNF-α and total nitrite levels. Additional mice were treated with LPS, as described previously, at the time of infection with each virus and their samples were processed identically. LPS-treated, uninfected mice, 3 per timepoint at 2, 12, 24 and 48 hours, and 3 untreated, uninfected mice were also killed and their sera were processed for TNF-α and nitrite analysis.

**2. Neuroinvasion by V3000 in Mice Treated with TNF-α Antibodies**

Because the data from experiment 1 showed some elevation of serum TNF-α in mice infected with V3000, suggesting that TNF-α might play a role in VEE virus neuroinvasion, this experiment was done to determine if treatment of mice with antibodies to TNF-α inhibited neuroinvasion by V3000. This was accomplished as follows. Mice were treated with goat polyclonal antibodies to mouse TNF-α (R&D Systems, Minneapolis, MD; Cat. # AF-410-NA), 2 _g each, subcutaneously at the time of infection. Mice were then anesthetized and antibody-treated and untreated controls were inoculated with 1000 pfu of V3000 in the left footpad. Mice were killed, 6 per group, at 24, 48, 72 and 120 hours PI. Brains were harvested and processed for virus titration. For those mice in the 120-hour group, a second TNF-α antibody treatment was performed 72 hours after the first treatment. The dose of TNF-α antibodies and treatment schedule were based on the methods of a previously reported study.[80]

**G. <u>The Effect of Tunicamycin on VEE Virus Replication In Vitro</u>**

To begin to understand the effect of TM on VEE virus replication, preliminary studies were performed using baby hamster kidney (BHK) cells. BHK-21 cells were grown as described subsequently. 8-well chamber slides and 6-well culture plates were seeded with BHK-21 cells and incubated for 12 hours at 37°C in 5% $CO_2$; individual wells were supplied with standard BHK-21 medium or medium containing 0.5 _g/ml of TM. At the time of infection, media were removed, wells were rinsed once with standard medium and wells were inoculated with V3000, diluted as described previously, at a multiplicity of

infection (MOI) of 1. Slides and plates were incubated for 1 hour, then all wells were washed once with sterile PBS and all wells were replaced with standard BHK medium. Slides and plates were further incubated for up to 48 hours. At 1, 6, 12, 24 and 48 hours PI, samples were harvested as follows. Medium was removed from the 6-well plates and stored at -85°C. The plates were then processed through 3 freeze-thaw cycles using dry ice to lyse cells and additional medium was removed and stored. These samples were later processed for virus titration. Medium was removed from all wells of the 8-well slides and the wells were washed in PBS. The chambers were removed and the slides were then placed in 10% neutral-buffered formalin and fixed for 24 hours. The slides were stained for VEE virus antigen as described subsequently and the numbers of antigen-positive cells per well of TM-treated and untreated wells were counted using a fluorescent microscope.

## H. Analytical Tests

### 1. Virus Titrations

For determination of virus titers, brain samples were homogenized in Eppendorf tubes with sterile pestles in volumes of sterile PBS containing Ca++/Mg++ and 0.1% bovine serum to produce 20% (weight/volume) suspensions. Blood samples were allowed to clot on ice. Clotted blood and brain suspensions were centrifuged for 5 minutes using a bench top centrifuge and sera and brain supernatants were removed. All samples were stored at -85º C until virus titration. Titration of VEE virus was performed by standard PA as previously described [21, 26] using BHK-21 cells (ATCC , Manasas, VA; Cat. #

CCL-10,). Briefly, BHK-21 cells were grown in Eagle's minimal essential medium supplemented with 10% tryptose phosphate broth, 0.29% L-glutamine and 10% fetal bovine serum. Cells between passages 55 and 65 were grown to approximately 80% confluency in 60 mm culture plates supplied with medium further supplemented by 100 units/ml penicillin and 0.05 mg/ml streptomycin. The medium was removed and plates were inoculated with 150 μl of 10-fold serial dilutions of virus samples. Plates were then incubated at 37°C in 5% CO2 for 1 hour. Three ml of medium containing 0.9% agarose was placed over the cell monolayer and plates were further incubated for 24 hours. Two ml of neutral red solution in 1X PBS with Ca++ and Mg++ was then added to each plate and the incubation was continued at 37°C for 5 hours or at 30°C overnight. Plaques were then counted for each plate. Additional plates were inoculated with serial dilutions of stock VEE virus of known titers as positive controls or with PBS as negative controls. All samples were run in duplicate.

**2. RNA Analysis**

For RNA extraction, approximately 100 μg of each brain sample was homogenized in Eppendorf tubes with 0.5 ml of Trizol (Life Technologies, Rockville, MD). An additional 0.5 ml Trizol was added to each tube and samples were then frozen at –80°C. Samples were later thawed and allowed to stand at room temperature (RT) for 5 minutes before the remainder of the RNA extraction was performed, according to the Trizol manufacturer's recommendations. After RNA extraction, the concentration and purity of RNA in each sample was determined using a spectrophotometer (Beckman Instruments, Inc., Columbia,

MD) by assessing the A260/280 ratios. Specimens were stored at -80°C until processing by ribonuclease protection assay (RPA) using the Riboquant® System (Pharmingen, San Diego, CA).

The RPA was performed as previously described.[38] Briefly, four multi-probe templates were used: rNT-1, mCK-2b, mCK-3b, and a custom designed template, to screen for the upregulation of specific genes (Table 2). 32P-labeled

Table 2

anti-sense RNA probes were synthesized by in vitro transcription and hybridized in excess to target RNA, after which free probe and other single-stranded RNA species were digested with RNases. The remaining 'protected' RNA probes were extracted, purified, and resolved on a denaturing 6% polyacrylamide gel. The gel was blotted, dried and exposed to Kodak X-OMAT(TM) AR film (Sigma, St. Louis, MO) for optimal exposure. Autoradiograms were scanned into a digital image and blots quantified by measuring pixel densities using Scion Image software for Windows (Scion Corporation, Frederick, MD). For genes of interest, expression relative to the constitutively-expressed glyceraldehyde-3-phophate dehydrogenase gene (GAPDH) was determined. Means (± 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.

**3. Determination of TNF-α and Total Nitrite Levels.**

Serum TNF-α levels were determined by solid phase ELISA using 96-well plates (Quantikine M, R&D systems, Minneapolis, MN.). Briefly, serum samples were added to wells coated with antibodies to recombinant mouse TNF-α

**Table 2. RPA Templates**.

| Templates | |
|---|---|
| *mCK-3b* | *Custom* |
| **Probes** | |
| IFN-γ | Fas Ag |
| IFN-β | Fas2L (TRAIL) |
| IL-6 | IL-1α |
| LTα | IL-1β |
| MIF | IL-1Ra |
| TGFβ1 | iNOS |
| TGFβ2 | TNFRp55 |
| TGFβ3 | TNFRp75 |
| TNF-α | L32 |
| TNF-β | GAPDH |
| L32 | |
| GAPDH | |

RNA probes included in the RNase protection assay standard and custom templates, as previously reported.[38] The listed probes were used to analyze the brains of TM-treated and untreated mice infected with virulent VEE virus. L32 and GAPDH are housekeeping genes used as internal controls.

and containing assay diluent. Plates were incubated for 2 hours at RT. Wells were washed 5 times with buffer, then horseradish peroxidase-conjugated secondary antibody was applied. Plates were incubated for 2 more hours at RT. Wells were again washed with buffer, then substrate chromogen (solution containing hydrogen peroxide and tetramethylbenzidine) was applied. Plates were further incubated for 30 minutes at RT in the dark, then stop solution (dilute hydrochloric acid) was applied. Optical densities were determined using a microplate reader (Benchmark, BIORAD, Hercules, CA) with the interference filter set to 450 nm and a correction filter set to 570 nm. Concentrations of TNF-α levels in serum samples were determined by plotting optical density readings against a standard curve generated from readings of a series of TNF-α standards. Recombinant mouse TNF-α was included in the assay as a positive control.

Serum total nitrite levels were measured by the Griess reaction using a commercially available kit (Calbiochem, San Diego, CA). Briefly, samples were placed into 96-well plate wells and buffer was added. Then, nitrate was reduced to nitrite by the sequential additions of nitrate reductase and NADH during a 20 minute incubation at RT. Color was developed by the addition of a solution containing sulfanilamide (p-aminobenzenesulfonamide) in HCl and N-(1-Naphthyl)ethylenediamine dihydrochloride (Griess Reagent) following a 5 minute incubation. Optical densities were determined using the microplate reader (BIORAD) with the interference filter set to 570 nm. Concentrations of nitrite levels in serum samples were determined by plotting optical density readings

against a standard curve generated from readings obtained from a series of nitrate standards.

**4. Histopathology and Immunohistochemistry**

Necropsies were performed immediately upon the death of each animal and harvested tissues were placed in 10% neutral-buffered formalin (NBF). After formalin fixation for a minimum of 3 weeks, tissues were placed into fresh NBF and removed from the BSL-3 suite. Tissues were then processed routinely and embedded in paraffin blocks. Five μm histologic sections were prepared, mounted on glass slides and stained with hematoxylin and eosin (HE). Duplicate sections of selected tissues were mounted on silane-coated slides (Sigma Diagnostics, St. Louis, MO) and labeled for VEE virus antigen by immunohistochemistry. Additional tissue sections were immunohistochemically-labeled for other markers, as described subsequently.

To detect VEE virus antigens in tissue sections, an immunoperoxidase method (Envision System, DAKO, Carpinteria, CA) was used according to the manufacturer's recommendations. Briefly, sections were deparaffinized and rehydrated to distilled water, then placed in citrate buffer (CB - 2.1 grams/liter, pH 6.0) at 97-100°C for 30 minutes for antigen retrieval. Sections were then washed in distilled water and blocked for endogenous peroxidase. Sections were incubated with the primary antibody, a rabbit polyclonal antiserum raised against VEE virus, eastern equine encephalitis virus, western equine encephalitits virus and Sindbis virus (provided by Cindy Rossi and Dr. George Ludwig, USAMRIID) diluted 1:5,000, for 30 minutes at RT. After washing with 3

changes of PBS, sections were incubated with peroxidase-labeled antibody against rabbit immunoglobulin (DAKO kit) for 30 minutes at RT. Color was developed by incubation in a solution containing 3,3'-diaminobenzidine and $H_2O_2$ (DAKO kit) for 7 minutes at RT. Additional sections were treated with nonimmune rabbit serum as negative controls. All tissue sections were counterstained with hematoxylin.

An immunofluorescent antibody (IFA) method was used to detect VEE virus antigens in cells infected in vitro. Formalin-fixed slides were washed in water and pretreated in CB at 97-100°C for 30 minutes for antigen retrieval. The alphavirus antibody, diluted 1:1,000 was applied and incubated at RT for 60 minutes. This was followed by a fluorescence-labeled secondary goat anti-rabbit immunoglobulin (ALEXA 488 or 594-labeled, Molecular Probes, Eugene OR) diluted 1:500 and incubated at RT for 1 hour. Coverslips were mounted using mounting medium containing DAPI or propidium iodide (Vectashield, Vector Labs, Burlingame, CA) for nuclear counterstaining and slides were observed under fluorescence microscopy using a Zeiss Axiophot system.

Fibrinogen immunohistochemistry was accomplished using a rabbit antibody to human fibrinogen (DAKO), diluted 1:500, by the immunoperoxidase method. Tissue sections were pretreated with Proteinase K (DAKO) for 6 minutes at RT prior to application of the primary antibody. The remainder of the immunoperoxidase procedure was perfomed as already described.

Double labeling methods were developed to detect VEE virus infection of neurons and astrocytes in the brains of mice. To detect virus infection of neurons,

brain sections were first labeled for VEE virus antigen by the IFA method (594 label). Then the sections were incubated with a fluorescence-conjugated marker for human neurons (anti-human neuronal protein HuC/HuD, 488-labeled; Molecular Probes) diluted 1:5 (20 g/ml) for 16-18 hours at 4°C. Sections were then coverslipped and observed under the fluorescence microscope. To detect virus infection of astrocytes, brain sections were first labeled for VEE virus antigen by IFA as described, except that antigen retrieval was accomplished using Tris-HCl buffer, 0.1M, pH – 8.0; 30 minutes at 97-100°C. Then the sections were incubated with a fluorescence-conjugated mouse monoclonal antibody to glial fibrillary acidic protein (GFAP, 488-labeled; Molecular Probes) diluted 1:100 (10 g/ml) for 16-18 hours at 4°C. Sections were then coverslipped and observed under the fluorescence microscope.

Selected brain sections of TM-treated and untreated mice infected with V3000 were stained by TUNEL to label apoptotic cells. These sections were stained using a commercially available kit (ApopTag, Serologicals, Norcross, GA) according to the manufacturer's recommendations. The kit was a peroxidase-based method and used diaminobenzidine as the chromogen. Sections were counterstained with methyl green.

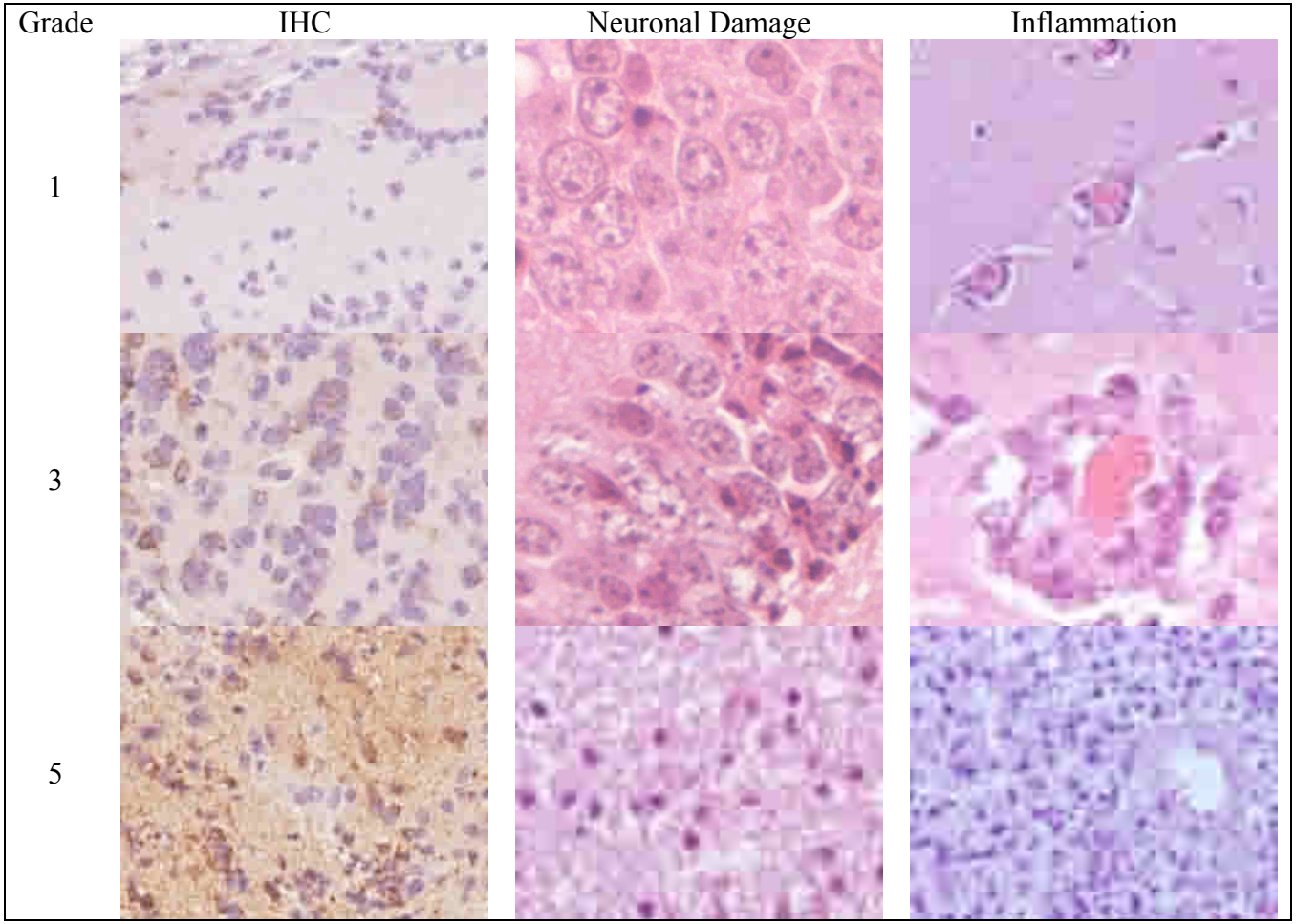
**5. Gradation of Histologic and Immunohistochemical Changes**

HE-stained and immunoperoxidase-labeled brain sections were observed by light microscopy and determinations of the amounts of virus antigen, inflammatory cell infiltrates and neuronal death were made using grading criteria applied to 9 different regions of the brain: olfactory bulbs, olfactory portion of the

brain proper, pyriform cortex, basal nuclei, diencephalon, hippocampus, neocortex, brainstem and cerebellum. Individual mouse mean scores were then determined as the sum of the scores from each of the brain regions divided by the number of the 9 regions present in the histologic sections of the case (all 9 regions were not always present on histologic sections for all cases) for each of the 3 parameters. From individual mean scores, group mean scores were determined as the sum of the individual mean scores of all mice in each experimental group at specific time points divided by 4 (4 mice per experimental group were examined at each time point except the 120 hour TM+/VEE+ group, for which only 1 mouse remained alive at this time). Positive scores were graded on a scale from 1 to 5 for inflammatory changes by the following criteria: 1 = minimal, a few perivascular cuffs and typically incomplete; 2 = mild, a few cuffs completely surrounding the vessels and a single cell layer thick; 3 = moderate, greater numbers of perivascular cuffs, complete and typically 2 cells thick but with no infiltration of the neuropil; 4 = marked, perivascular cuffs with mild to moderate infiltration of the neuropil; 5 = severe, infiltration of inflammatory cells essentially effaces the neuropil. For both the amount of antigen and the amount of neuronal cell death, positive scores were graded on a scale from 1 (generally 5 cells or fewer in a given region are affected) to 5 (all or nearly all cells in a given region are affected). Examples of some of these gradations are provided in Figure 2.

Fig. 2

**Figure 2. Examples of immunohistochemical (IHC) and histological grades**. The photomicrographs shown illustrate the appearance of immunohistochemical and histopathological changes that correspond to graded parameters for the amounts of viral antigen, neuronal damage and inflammation (see materials and methods) used for TM-treated and untreated mice infected with virulent VEE virus. Positive scores were graded from 1 to 5 for inflammatory changes by the following criteria: 1 = minimal , a few perivascular cuffs and typically incomplete; 2 = mild, a few cuffs completely surrounding the vessels and a single cell layer thick; 3 = moderate, greater numbers of perivascular cuffs, complete and typically 2 cells thick but with no infiltration of the neuropil; 4 = marked, perivascular cuffs with mild to moderate infiltration of the neuropil; 5 = severe, inflammatory cells nearly efface the neuropil. For both the amount of antigen and the amount of neuronal damage (necrosis and/or apoptosis), positive scores were graded on a scale from 1 (generally 5 cells or fewer in a given region are affected) to 5 (all or nearly all cells in a given region are affected). The photomicrograph illustrating inflammation grade "5" was taken from a VEE-infected mouse in an unrelated experiment. All other photomicrographs were taken from the current studies.


Grade
IHC
Neuronal Damage
Inflammation
1
3
5

**6. Electron microscopy**

Mice were treated with 2.5 μg of TM as described previously. Twenty four hours later, the treated and untreated control mice were deeply anesthetized and killed by cervical dislocation. Brains were removed immediately following death and 1 mm cubes of pyriform cortex, neocortex and thalamus were placed in universal fixative (4% paraformaldehyde, 1% glutaraldehyde) for 2 hours at RT. Following fixation, all samples were washed 3 times in 0.1 M Millonig's phosphate buffer (pH 7.4) and processed for standard transmission electron microscopy. Briefly, tissues were postfixed in 1% osmium tetroxide, dehydrated in ethanol and propylene oxide and embedded in EMbed-812 resin (Electron Microscopy Sciences, Warrington PA). Ultrathin sections were cut, placed on 200-mesh copper grids, stained with uranyl acetate and lead citrate and examined with a Phillips CM100 transmission electron microscope. Images were photographed with an AMT side-mounted, Kodak-Megaplus Camera.

**7. Statistical analysis.** Data were analyzed using a commercially-available software program, SPSS for Windows version 10.0 (SPSS Inc., Chicago, IL). In the mortality studies, data for percentage mortality and MST were compared between groups treated with individual chemicals and the corresponding untreated groups using the *t*-test for independent samples. In the pathogenesis studies, data obtained for virus titers, viral antigen, neuronal necrosis and brain inflammation were compared for TM-treated and untreated mice at equivalent time points also using the independent *t*-test. In other studies, virus titers were compared for treatment and control groups at equivalent time points by the *t*-test and in selected

cases, for multiple time points by multiple regression analysis. In the TM pathogenesis study with V3000 inoculated in the footpad, the mouse body weights were compared between all 4 experimental groups by one-way analysis of variance (ANOVA) using the Tukey post-test. In the study involving serum TNF-$\alpha$ and NO levels of mice infected with V3000 or V3034, results for infected mice were compared to those of uninfected control mice at individual time points by the independent *t*-test. Statistical significance was established at $P < 0.05$ a priori. All analyses were two-tailed tests.

## III. RESULTS

### A. Mortality Studies

The effect of tunicamycin (TM) , lipopolysaccharide (LPS) and pyridostigmine bromide (PB) on the survival times of mice infected with virulent VEE virus was determined in a series of mortality studies in which mice were infected with V3000 in the footpad. As Table 3 demonstrates, all groups of mice infected with V3000 showed 100% mortality. However, the mean survival time

Table 3

(MST) of the TM-treated mice, at 7.3 days, was significantly less ($P < .05$) than that of the corresponding untreated mice, at 9.9 days. In the LPS and PB mortality studies, differences in the MSTs of groups of mice infected with V3000 after treatment with either chemical were not significantly different from untreated control groups. In the case of PB, multiple experiments were done in which the timing of PB administration was altered. Mice were treated 24 hours before infection in experiment 1, essentially at the time of infection in experiment 2 and at the time of expected peak viremia in experiment 3. Yet in all 3 experiments, there was little difference in the MST of treated versus untreated mice. Pyridostigmine administration also failed to produce any significant increase in the mortality of mice infected in the footpad with the attenuated strain V3010, although 1 of 10 mice in the PB-treated group died, compared to none of the 10 untreated mice. Pyridostigmine bromide did, however, increase the mortality of mice infected with sublethal doses of two other encephalitic viruses. Groups of mice treated with low doses of PB each demonstrated 50% mortality

following infection with SFV and EMCV, compared to 0% in both groups of untreated mice. These differences were statistically significant.

## B. Effect of Tunicamycin Administration on the Pathogenesis of VEE

Subsequent to the mortality study showing that TM significantly decreased the MST of mice infected with virulent VEE virus, a series of experiments was performed to attempt to determine by what means TM enhanced the pathogenicity of VEE virus.

### 1. V3000 Pathogenesis Following Footpad Infection

First, a serial sacrifice study was performed and several virological and pathological parameters were measured in order to broadly characterize the effects of TM on the course of disease caused by virulent VEE virus in mice infected peripherally.

#### a. Clinical Signs

After footpad infection withV3000, mice treated with TM (TM+/VEE+) developed clinical signs sooner than untreated mice (TM-/VEE+). These signs were manifest as progressive weight loss (Figure 3), lethargy, huddling and dehydration observed at 72 hours post inoculation (PI) in the TM+/VEE+ mice and at 96 hours PI in the TM-/VEE+ mice. Infected mice later developed additional clinical signs including spastic tremors and hindlimb paresis or paralysis. Three of the 4 TM+/VEE+ mice died between 96 and 120 hours PI.

Figure 3

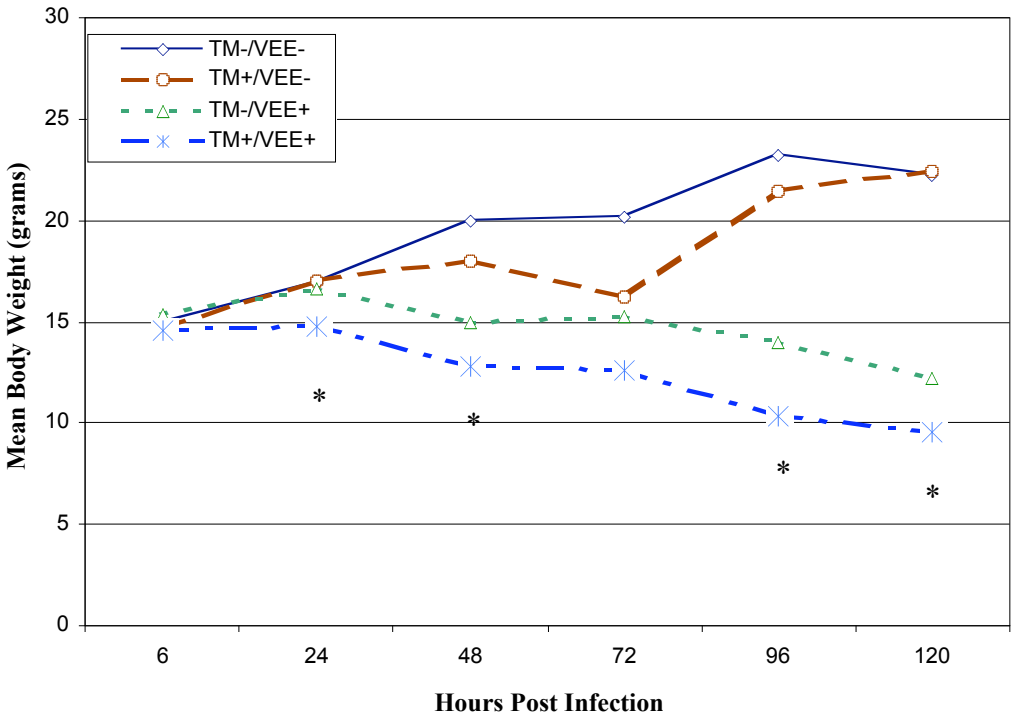



**Figure 3. Body weights in tunicamycin-treated and untreated mice infected with virulent VEE virus.** Six-to-8-week-old, male, CD-1, TM-treated (TM+) or untreated mice (TM-) were infected with 1 x $10^3$ pfu of V3000 virus (VEE +) in the footpad. Mice were sacrificed (4 per group) and weighed immediately after death at the indicated times post infection (mice in the 12-hours group were not weighed). TM-treated and untreated, uninfected (VEE -) mice (2 per group) were also included. The mean weights for TM+/VEE+ mice were significantly different than those in the corresponding TM-/VEE+ mice at the indicated times (*). The mean weights of the TM+/VEE- mice were not significantly different than those of the TM-/VEE- mice at all times.

The only clinical change evident in uninfected mice treated with tunicamycin (TM+/VEE-) was somewhat less rapid weight gain than uninfected, untreated mice (TM-/VEE-).

**b. Virus Titers in Sera and Brains**

TM-treated and untreated mice exhibited nearly identical levels of VEE virus in the blood following footpad infection with V3000 (Figure 4). In both groups of mice, detectable virus was present at 6 hours PI and virus titers peaked at 24 hours PI before declining progressively until the end of the study.

Figure 4

Differences in the virus levels in the brain, however, were apparent between the two groups (Figure 5). The geometric mean virus titer in the brains of TM-treated mice was much higher than in untreated mice at 48 hours PI. This difference was statistically significant. Virus levels of TM-treated mice also exceeded those of untreated mice at later time points until 120 hours PI, though these differences were not statistically different. The virus levels detectable at 24 hours PI for TM-treated and untreated mice did not differ significantly, however only a few mice had detectable virus titers at this time.

Figure 5

**c. Virus Infection and Spread in the Brain**

Immunohistochemistry showed that VEE virus antigen first appeared in the brains of 3 of 4 TM-treated mice at 24 hours PI (Figure 6). At this time, antigen was present primarily in the olfactory bulbs and olfactory associated regions of the brain proper such as olfactory nuclei and the lateral olfactory tracts.

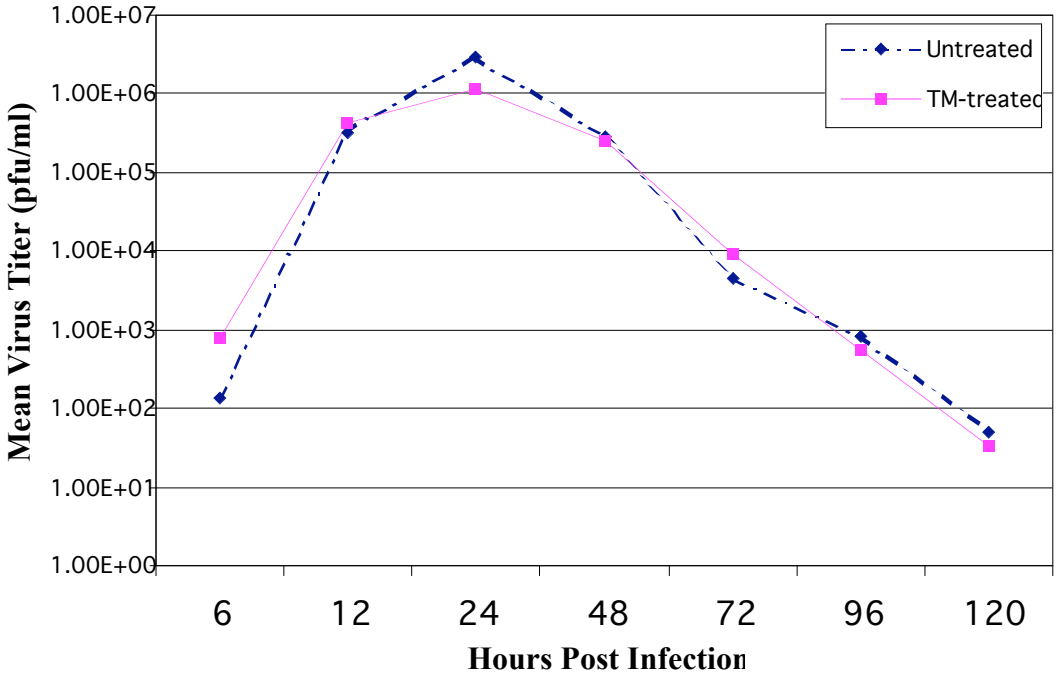



**Figure 4. Serum titers of virulent VEE virus in tunicamycin-treated and untreated mice.** TM-treated and untreated mice were infected with 1 x $10^3$ pfu of V3000 virus in the footpad. Mice were sacrificed (4 per group) at the indicated times post infection. Serum samples were obtained and analyzed by plaque assay. The sensitivity of the plaque assay was 33 plaque forming units (pfu)/ml.

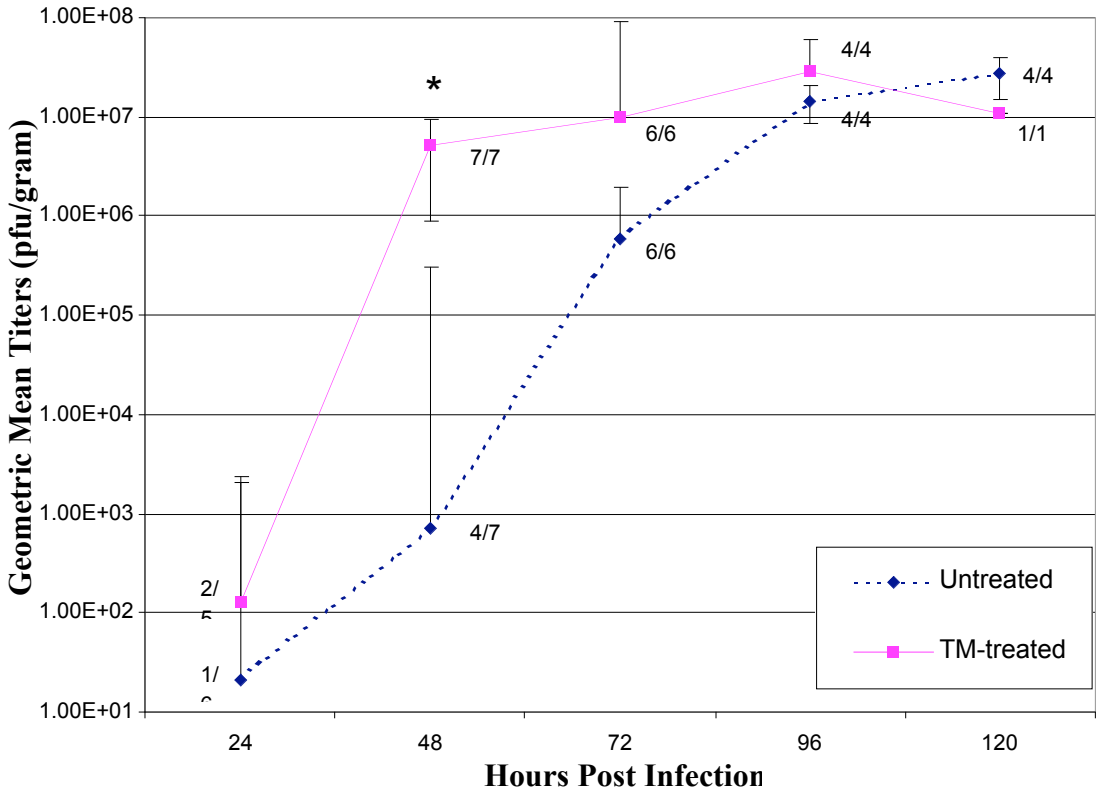



**Figure 5. Titers of virulent VEE virus in the brains of tunicamycin-treated and untreated mice.** TM-treated and untreated mice were infected with 1 x $10^3$ pfu of V3000 virus in the footpad. Mice were sacrificed at the indicated times post infection. Brain homogenates were obtained and analyzed by plaque assay. The fractionated numbers for each data point represent the number of positive mice divided by the number of total mice at that time. The mean brain titer of TM-treated mice was significantly greater than that of untreated mice at 48 hours (*). In the TM-treated group, only 1 of 4 mice remained alive at 120 hours PI. The sensitivity of the plaque assay was 165 plaque forming units (pfu)/gram. Data were obtained using mice in 3 separate experiments.

Figure 6

By 48 hours PI, at least a few antigen-positive cells were present in all regions of the brain, although the olfactory regions and the thalamus contained the most abundant amounts of antigen. In untreated mice, virus antigen did not appear in the brain until 48 hours PI. Virus antigen also first appeared in the olfactory structures of these mice and spread to all regions of the brain by 72 hours PI.

The amounts of antigen accumulated progressively throughout the time course of the study in both TM-treated and untreated mice. At any given time point, the amount of antigen present in a particular region of the brain was typically greater in TM-treated mice than in untreated mice (Figure 7).

Figure 7

No differences in the pattern of virus spread throughout the brain was evident in TM-treated versus untreated mice. The immunohistochemistry results indicated that VEE virus invaded the brains of mice in both groups via the olfactory apparatus and spread throughout the brains of mice in a fashion consistent with previous reports.[28, 42] Antigen was not evident within endothelial cells and there was no perivascular pattern of antigen deposition in either TM-treated or untreated mice, as would be expected if VEE virus entered the brain directly from the bloodstream; i.e. through the blood-brain barrier (BBB). In both TM-treated and untreated mice, VEE virus antigen was present largely in neurons. The identity of these cells was confirmed by double immunolabeling for VEE virus antigen and a neuronal marker (Figure 8).

Figure 8

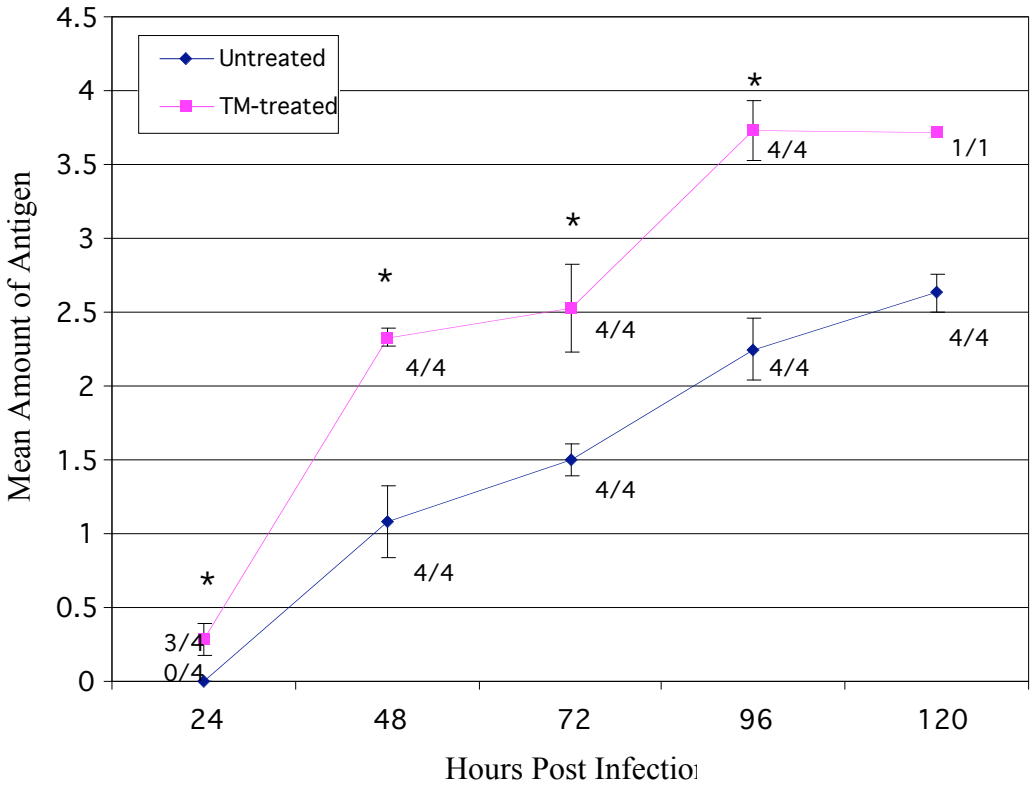
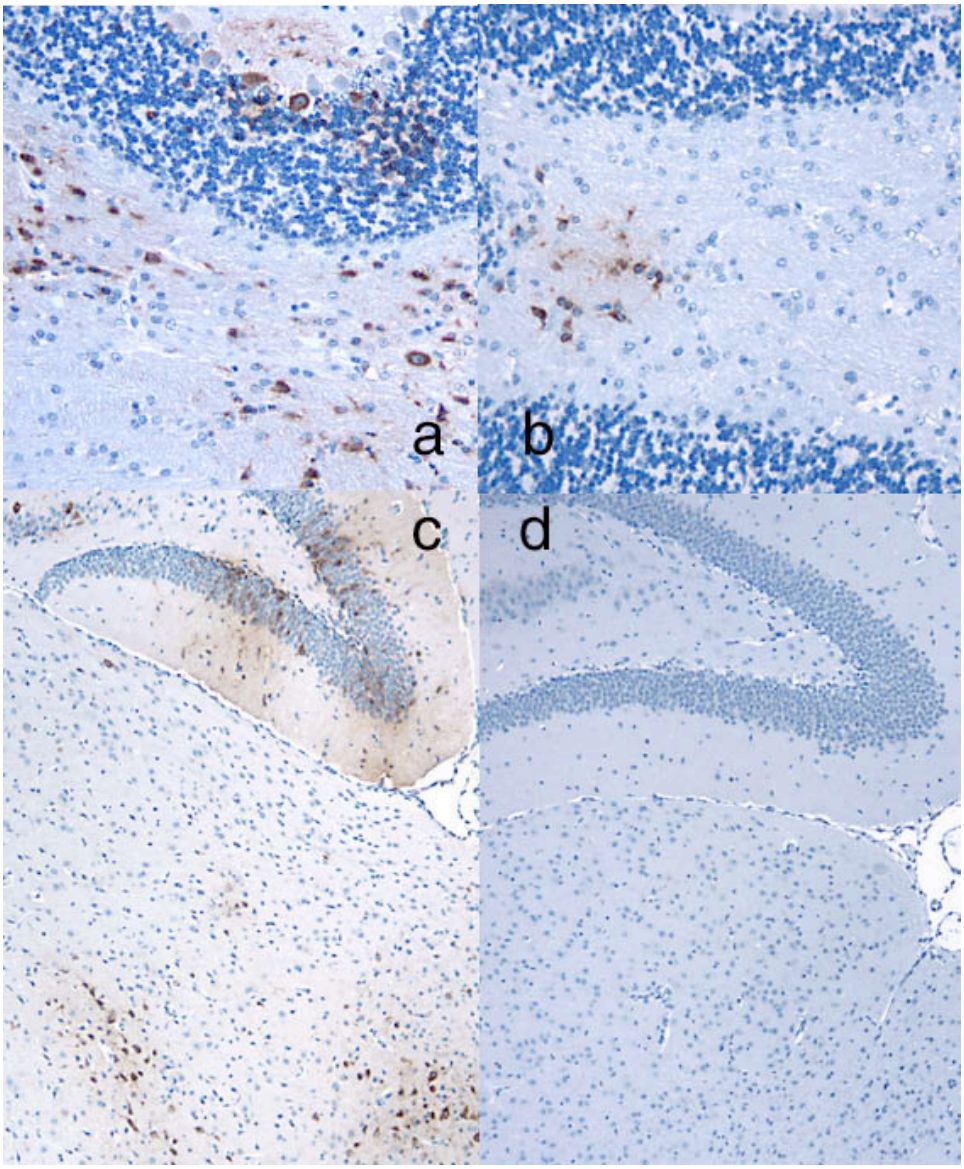



**Figure 6. Semiquantitative comparison of virus antigen in the brains of tunicamycin-treated and untreated mice infected with virulent VEE virus.** TM-treated and untreated mice were infected with 1 x $10^3$ pfu of V3000 virus in the footpad. Mice were sacrificed (4 per group) at the indicated times post infection. Brain samples were formalin-fixed and processed for immunohistochemistry using a rabbit polyclonal antibody that recognizes VEE virus. Immunostained sections were scored as described in the materials and methods section. The fractionated numbers for each data point represent the number of positive mice divided by the number of total mice at that time. The mean antigen scores of TM-treated mice were significantly greater than those of untreated mice at the indicated times (*). In the TM-treated group, only 1 of 4 mice remained alive at 120 hours PI.

**Figure 7. VEE virus antigen in tunicamycin-treated and untreated mice infected with virulent VEE virus**. Samples were obtained from the mice described in figure 7 and processed as stated. Virus antigen is relatively abundant in the cerebellum of a TM-treated mouse 72 hours post infection (a); in contrast, antigen is limited in the cerebellum of an untreated mouse (b). The hippocampus and thalamus of a TM-treated mouse also contained significant amounts of antigen at 48 hours PI (c), while little or no antigen was present in the corresponding regions of an untreated mouse (d). Histologic sections were stained by an immunoperoxidase method using a rabbit antibody to VEE virus; hematoxylin counterstain.


a
b
c
d

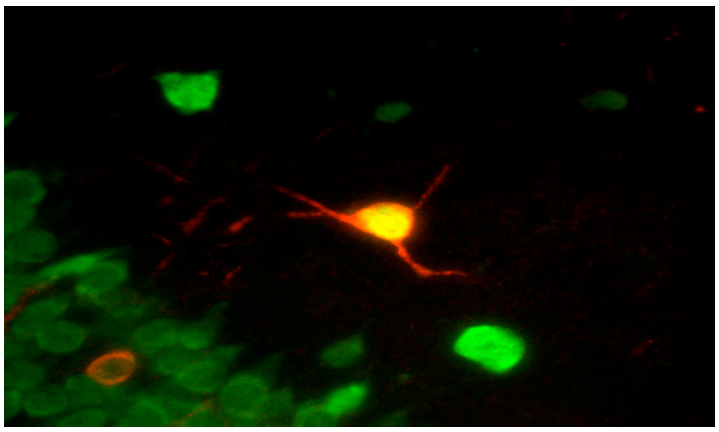


**Figure 8. VEE virus infection of neurons.** The brain of a mouse infected with virulent VEE virus in the footpad, using double immunofluorescence labeling for VEE virus antigen (red) and neuronal protein (green) demonstrates virus infection of a Purkinje neuron (combined antigens expressed as yellow) and a neuron in the granular layer of the cerebellum. Note the presence of virus antigen in the cell bodies of both neurons and the neuritic processes of the Purkinje neuron. Immunofluorescence antibody method using a rabbit antibody to VEE virus and a fluorochrome-labeled mouse antibody to human neuronal protein (Hu C/D).

Only rarely were cells identified as astrocytes observed to contain VEE virus antigen in tissue sections double labeled for VEE antigen and GFAP (Figure 9). No differences in the antigen-labeling patterns of neurons and astrocytes were evident between TM-treated and untreated mice.

Figure 9

**d. Histopathologic Changes in the Brain**

The histopathologic findings in mice infected with virulent VEE virus were also consistent with those of previous reports.[28, 42] Inflammatory cell infiltrates (Figure 10) and neuronal cell death (Figure 11) were evident in the brains of TM-treated mice at 48 hours PI and in untreated mice at 72 hours PI.

Figure 10

Figure 11

Similar to the appearance of virus antigen, histologic lesions were essentially limited to the olfactory portions of the brain at the earliest time points. At later times, lesions appeared in other regions of the brain in a pattern that paralleled that of viral spread. The appearance of histologic changes generally lagged behind the appearance of virus in particular regions of the brain by about 24 hours. Also similar to the immunohistochemistry findings, at any given time point, inflammation and neuronal cell death were more severe in TM-treated mice than in untreated mice.

The earliest inflammatory lesions were characterized by margination of leukocytes and a few perivascular cuffs that were either incomplete or were composed of a single layer of cells, primarily lymphocytes and some neutrophils

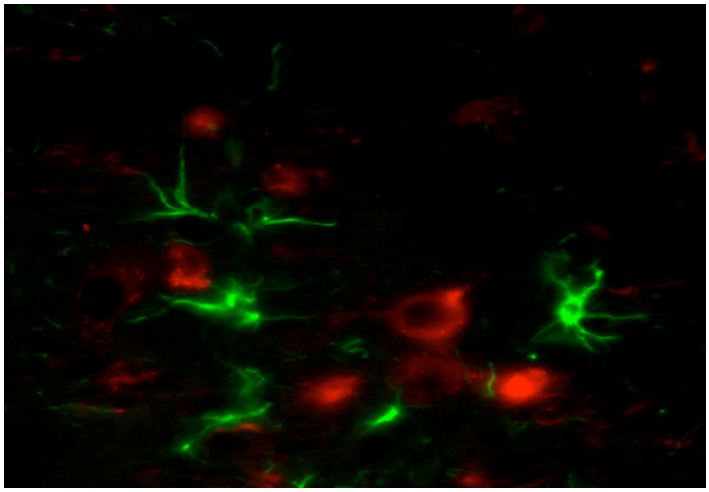


**Figure 9. Double IFA-labeling for VEE virus infection of astrocytes.** The brain of a mouse infected with virulent VEE virus in the footpad, using double immunofluorescence labeling for VEE virus antigen (red) and glial fibrillary acidic protein (GFAP - green) demonstrates that virus-positive cells are GFAP negative and GFAP-positive astrocytes are virus antigen-negative. Immunofluorescence antibody method using a rabbit antibody to VEE virus and a fluorochrome-labeled mouse antibody to GFAP.

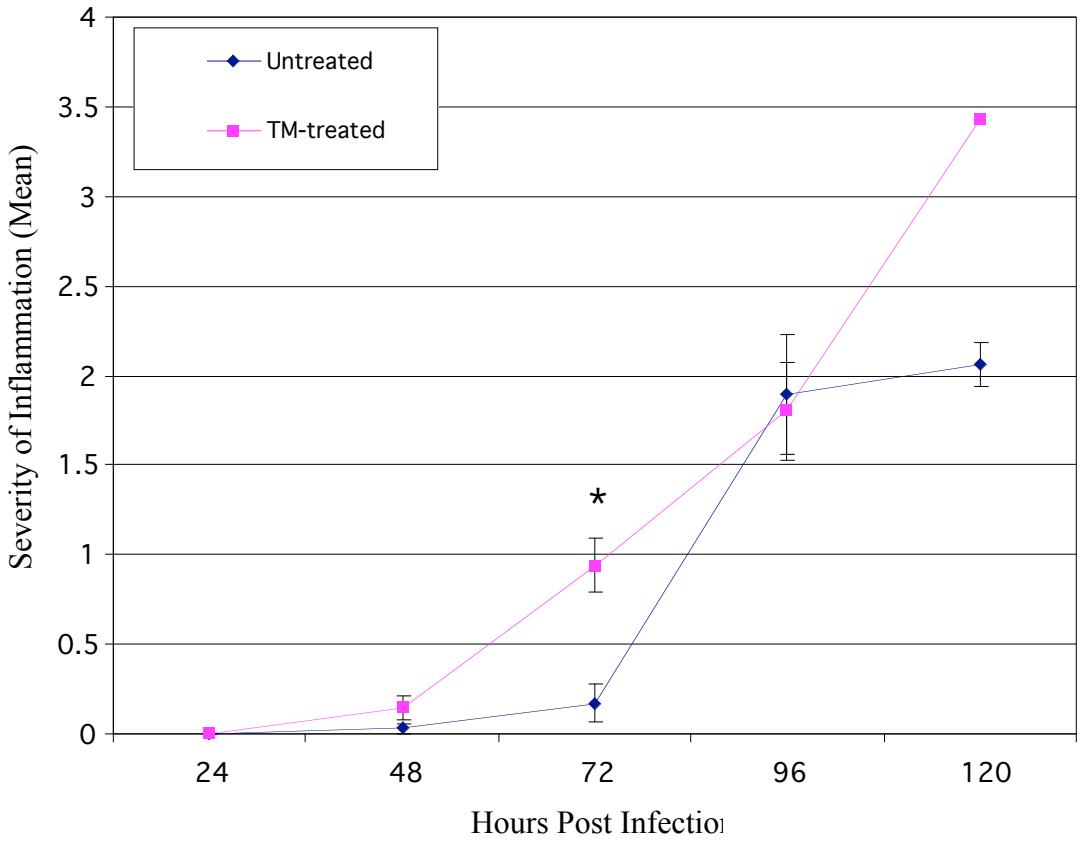



**Figure 10. Semiquantitative comparison of inflammation in the brains of tunicamycin-treated and untreated mice after footpad infection with virulent VEE virus.** Mice were treated as described in figure 7 and brain sections were scored for inflammation as described under materials and methods. The mean scores of TM-treated mice were significantly greater than those of untreated mice at 72 hours PI (*). In the TM-treated group, only 1 of 4 mice remained alive at 120 hours PI.

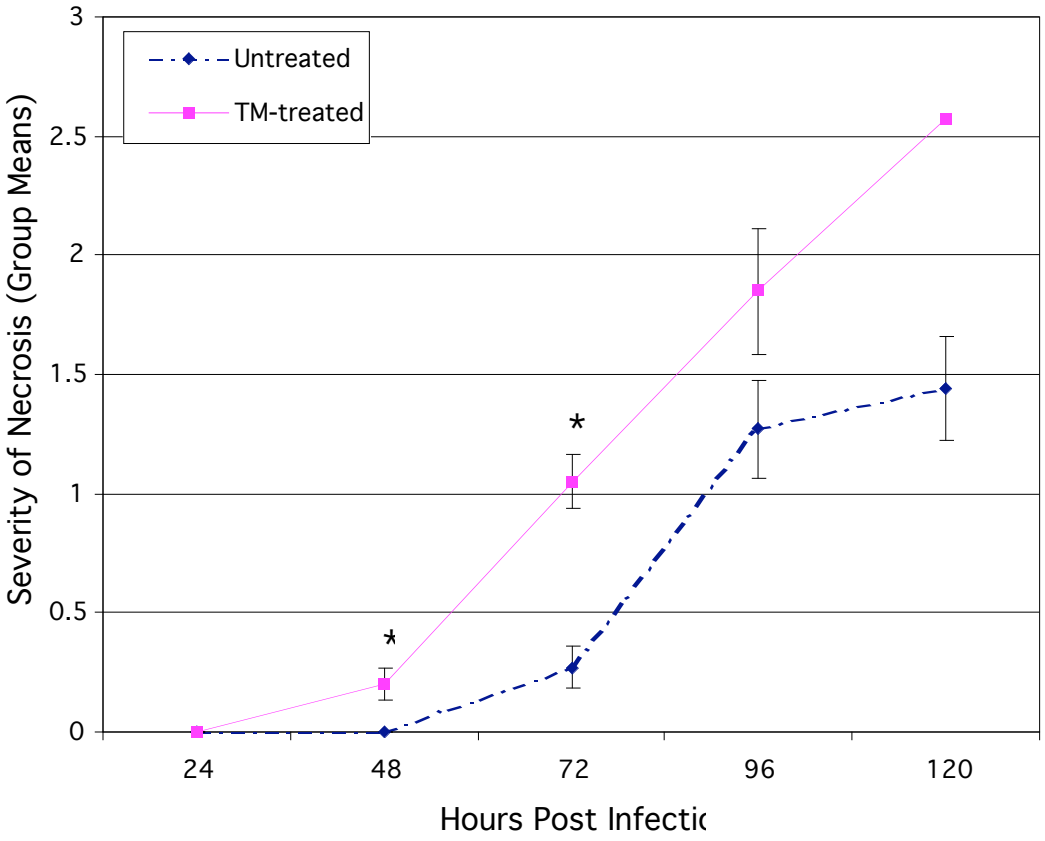



**Figure 11. Semiquantitative comparison of neuronal death in the brains of tunicamycin-treated and untreated mice after footpad infection with virulent VEE virus.** Mice were treated as described in figure 7 and brain sections were scored for neuronal necrosis and apoptosis as described under materials and methods. The mean scores of TM-treated mice were significantly greater than those of untreated mice at 48 and 72 hours PI (*). In the TM-treated group, only 1 of 4 mice remained alive at 120 hours PI.

and macrophages. More advanced inflammatory lesions consisted of more numerous perivascular cuffs of greater thickness and there was also infiltration of inflammatory cells into the surrounding neuropil. Gliosis, characterized by the presence of activated microglial cells and activated astrocytes, was also present in more advanced inflammatory foci.

Morphologically, neuronal damage appeared to be manifest as both necrosis and apoptosis (Figure 12A). In areas such as the pyramidal layer of neurons in the pyriform cortex, a consistent early site of infection in the brain, neurons typically maintained their shape and cell borders, had brightly

Figure 12

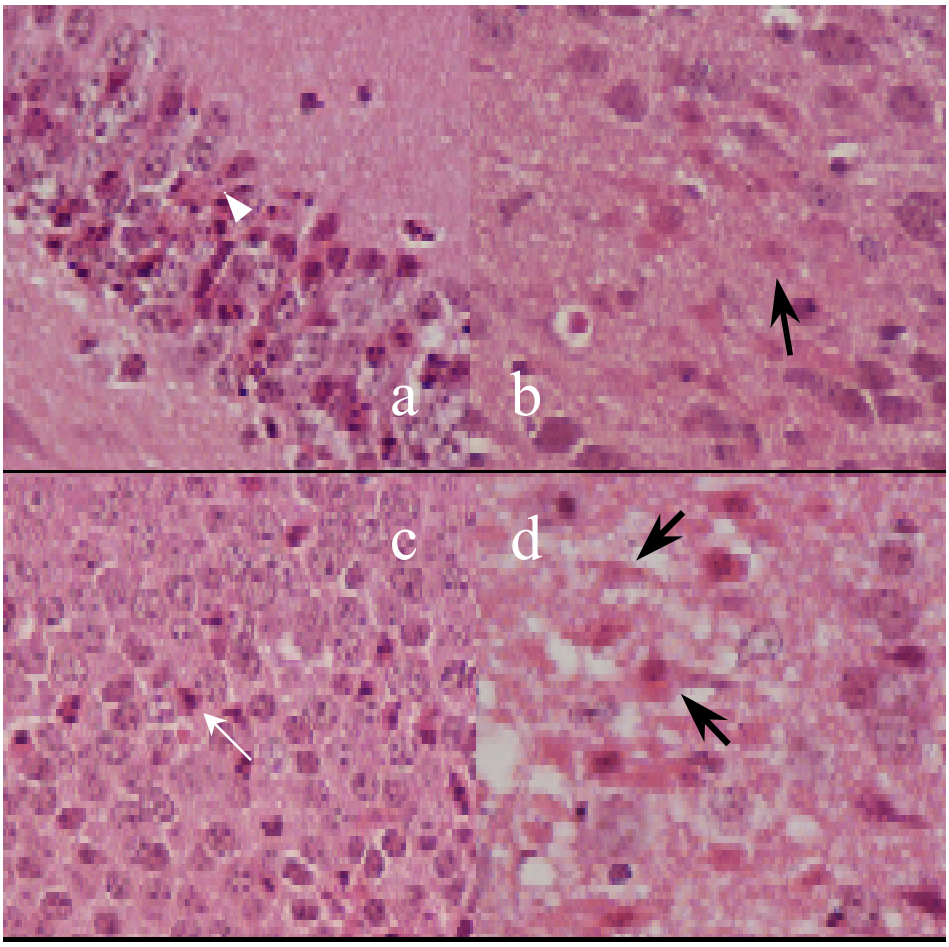
eosinophilic cytoplasm and faded to poorly discernible nuclei (karyolysis), i.e. features typical of necrosis (Figure 12A; b,d). Similar features were characteristic of other types of large neurons, including Purkinje neurons in the cerebellum and neurons in the cerebral cortex. Conversely, affected hippocampal neurons and granule cells of the cerebellum were often shrunken and contained condensed or segmented nuclei (pyknosis) or were fragmented into apoptotic bodies (Figure 12A; a,c). Apoptotic cells which were likely inflammatory cells were also evident in foci of inflammation in advanced lesions. Neither neuronal damage, inflammation nor other histologic changes were evident in the brains of TM-/VEE- and TM+/VEE- mice at any time points.

Unexpectedly, neurons with features of either necrosis or apoptosis were similarly labeled using the TUNEL method (Figure 12B). TUNEL-labeled portions of brain sections were essentially limited to those that contained neurons

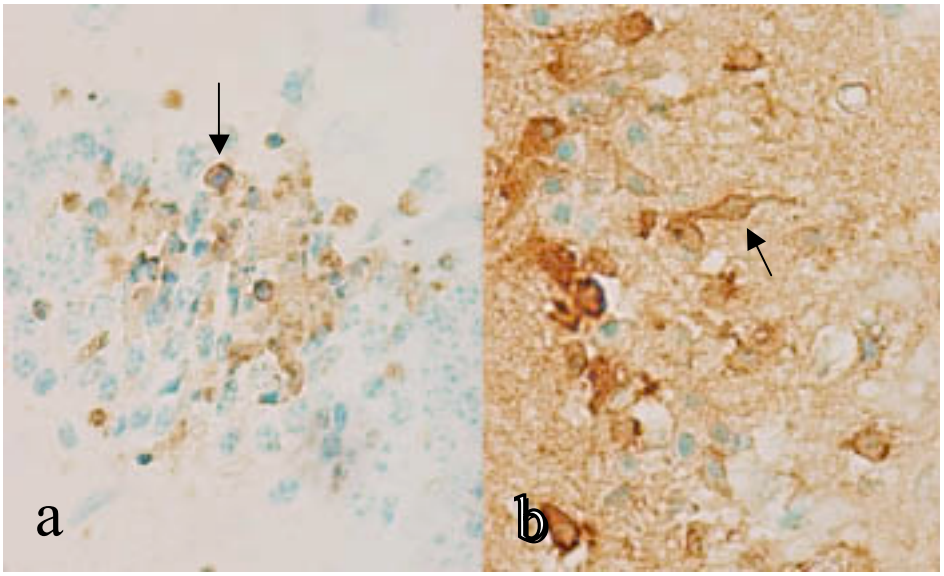
**Figure 12. Neuronal necrosis and apoptosis in mice infected with virulent VEE virus.** Features of necrosis and apoptosis are evident in HE-stained (panel A) and TUNEL-positive (panel B) regions of brain.

Panel A: In the hippocampus (a,c), affected neurons are shrunken and rounded and have condensed chromatin (white arrow), segmented nuclei, and form apoptotic bodies (white arrowhead); i.e. features of apoptosis. In the pyriform cortex (b,d), damaged pyramidal neurons generally maintain their shape and cell borders, but have bright eosinophilic cytoplasm and pale to nondiscernible nuclei (karyolysis - black arrows); these are features typical of necrosis. Sections are from TM-treated (a,c,d) and untreated (b) mice at 72 (a) or 96 (b,c,d) hours post infection. Hematoxylin and eosin stain.

Panel B: TUNEL-positive neurons are present both in the hippocampus (a) and pyriform cortex (b) of a TM-treated mouse infected with V3000. Note, however, that affected cells show labeling of primarily cytoplasm and that nuclear material is often not labeled (arrows) in both regions of the brain. TUNEL-peroxidase method with diaminobenzidine chromogen, methyl green counterstain.




A




B

with the morphological changes already described. TUNEL-labeling was most prominent in the cytoplasm of cells, including neuritic processes. In some severely affected foci of neuronal damage, such as the olfactory bulbs and pyriform cortices, the neuropil was also labeled. In many TUNEL-positive neurons, the nuclei did not appear to be labeled.

**e. Blood brain Barrier Damage**

Immunohistochemistry to detect fibrinogen was used to determine if leakage of this blood protein from blood vessels in the brain indicative of BBB damage occurred in TM-treated and untreated mice infected with VEE virus (Figure 13). The most conspicuous evidence of BBB damage was seen in the

Figure 13

brains of mice at later timepoints. In these mice, there was abundant extravascular fibrinogen evident as diffuse labeling of regions of the brain in which virus was detected early and in which tissue damage was extensive. Consistently, the olfactory bulbs, olfactory lobes and pyriform cortices exhibited extensive fibrinogen leakage around vessels at days 3, 4 and 5 PI in TM-treated mice and at days 4 and 5 PI in untreated mice. Less frequently, some portions of the neocortex also exhibited diffuse immunolabeling at these times. Within areas of diffuse labeling, there was often more intense fibrinogen-labeling immediately surrounding some small blood vessels. Similar perivascular labeling was also present in remaining regions of the brain in which diffuse labeling was not present. Some of the vessels from which fibrinogen leakage was apparent were lined by swollen endothelial cells and exhibited leukocyte margination or

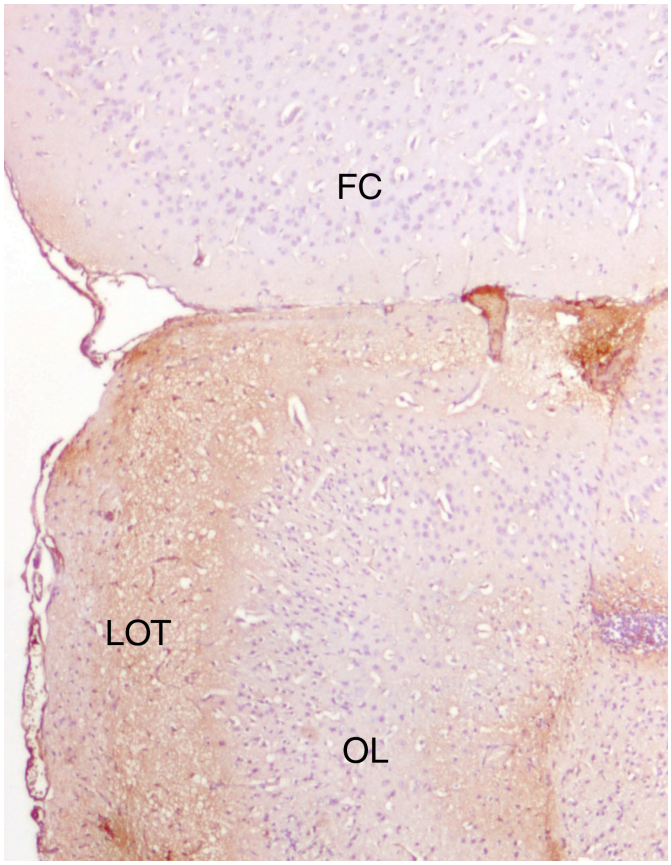



**Figure 13. Fibrinogen leakage in the brain of a mouse infected with virulent VEE virus.** Using an antibody to fibrinogen, abundant leakage of plasma proteins is indicated by near diffuse staining of the olfactory lobe (OL), particularly the lateral olfactory tract (LOT), whereas the frontal cortex (FC) exhibits only limited staining. This mouse was treated with tunicamycin, then inoculated with V3000 and killed 72 hours PI. Immunoperoxidase method using a rabbit antibody to human fibrinogen; hematoxylin counterstain.

perivascular cuffs. However, similar features were also characteristic of some vessels from which no fibrinogen leakage was evident.

In a few TM-treated and untreated mice as early as 2 hours PI, there was perivascular fibrinogen labeling of occasional vessels, in particular the small vessels of the neocortex. Some vessels in other regions of the brain were also affected. This pattern of perivascular fibrinogen labeling was seen in the brains of 2 of 4 untreated mice and 1 of 4 TM-treated mice at 2 hours PI, 1 of 2 untreated mice and 2 of 4 TM-treated mice at 12 hours PI and all of the mice at 24 hours PI. Intravascular fibrinogen labeling provided internal positive controls for all brain sections studied. Extravascular labeling was not evident in the brain sections of negative control mice or uninfected mice treated with TM.

**f. Regulation of Cytokines in the Brain**

The RNase protection assay (RPA) was used to demonstrate the expression of messenger RNAs of a variety of cytokines and other immunological/inflammatory mediators in the brains of mice in this experiment. The results of RPA analysis showed more rapid upregulation of the mRNAs of several important cytokines in the brains of TM-treated mice compared to untreated mice (Figure 14). Upregulation of IL-6 (greater than 5X versus untreated mice) was evident at 24 hours PI in TM-treated mice, compared to only

Figure 14

a slight upregulation at 72 hours PI in untreated mice. TNF-□, IFN-□, FAS-a, IL-1_, and IL-1Ra were all upregulated at 48 hours PI (at levels of 5X or more versus controls) in TM-treated mice but were not upregulated until 72 hours PI in the

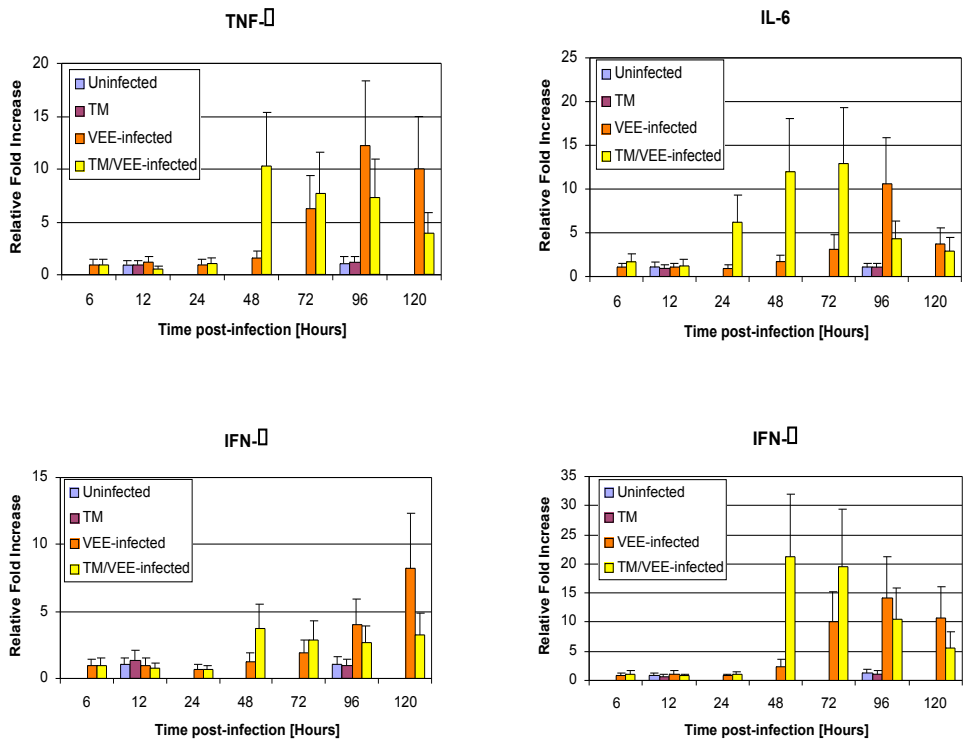



**Figure 14. Expression of the mRNAs of important cytokines in the brains of tunicamycin-treated and untreated mice infected with virulent VEE virus**. The mRNAs of several key cytokines are upregulated at 24 (IL-6) and 48 (TNF-_, IFN-_, IFN-_) hours post-infection in the brains of TM-treated mice infected with V3000, preceding upregulation of the corresponding factors in the brains of untreated mice. Mice were infected with V3000 in the footpad and brain samples were analyzed by RNase protection assay (RPA). The data presented represent the group means of individual factors expressed as relative fold increase over internal controls.

untreated mice. IFN-β, iNOS, TNFRp75, FAS2L and TNFRp55 also showed upregulation at 48 hours PI in TM-treated mice, but the degree of upregulation (2 to 4X versus controls) was not as great as that of the other cytokines on the basis of relative fold increase.

**2. Tunicamycin and Replication of Virulent VEE Virus in the Brain**

The virological and immunohistochemical results of the first pathogenesis experiment indicated that virulent VEE virus invaded the brains of TM-treated mice earlier than in untreated mice. To rule out the possibility that virus entered the brain at essentially the same time in TM-treated and untreated mice but then replicated much faster in TM-treated mice, virus titers were measured at multiple time points following intracranial (IC) inoculation of treated and untreated mice. In both groups of mice in this experiment, V3000 appeared sporadically in the hemisphere contralateral to the one into which virus was inoculated at 12 to 24 hours PI (Figure 15). At subsequent time points, virus was present consistently in the contralateral brain hemispheres of all mice and the virus kinetics were nearly identical between the groups of TM-treated and untreated mice, peaking at 96 hours PI in both cases. Essentially, TM had no apparent effect on virulent VEE virus replication in the brain.

Figure 15

**3. Tunicamycin and Neuroinvasion by V3034** The results of the IC inoculation experiment provided additional evidence that TM specifically increased neuroinvasion by virulent VEE virus. To further confirm this notion, the ability of an attenuated VEE virus, previously shown to exhibit limited

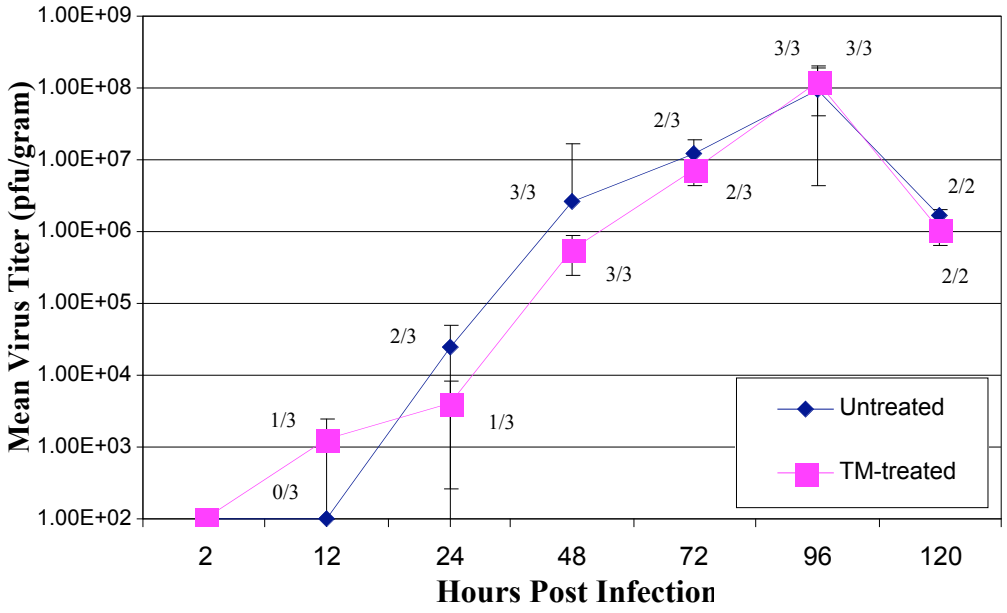



**Figure 15. Virulent VEE virus replication in the brains of tunicamycin-treated and untreated mice (intracranial inoculation).** TM-treated and untreated mice inoculated with V3000 in the left cerebral hemisphere show very similar virus replication profiles in the right cerebral hemisphere throughout the timecourse of the study. Mice were treated with TM 24 hours prior to inoculation of 1 x $10^3$ pfu of V3000. The fractionated numbers for each data point represent the number of positive mice divided by the number of total mice at that time. Only 2 of 3 mice in each group remained alive at 120 hours PI. The sensitivity of the plaque assay was 165 plaque forming units (pfu)/gram.

neuroinvasion,[26] to invade the brains of TM-treated mice after peripheral infection was tested. After footpad inoculation of the molecular clone V3034, neuroinvasion was evident by plaque assay at 48 hours in all 4 mice treated with TM, compared to none of the 8 untreated mice killed at this time (Figure 16). At

Figure 16

72 and 96 hours PI, 3/4 and 4/4 untreated mice, respectively had detectable virus compared to 4/4 and 4/4 TM-treated mice at these times. The mean titers of the TM-treated mice, though, were more than 100-fold greater than those of untreated mice at these times. These differences were statistically significant.

V3034 was consistently present in the serum of treated and untreated mice at 12 and 24 hours PI. Importantly, the numbers of viremic mice and the levels of virus in the blood of these mice did not appear to differ between TM-treated and untreated mice (Figure 17).

Figure 17

**C. Tunicamycin-induced Changes in the Blood-brain Barrier.**

In a limited electron microscopy study, capillary damage was evident in the brains of 2 mice 24 hours after treatment with TM. The most dramatic change was the presence of edema surrounding some capillaries (Figure 18). Swollen astrocytic

Figure 18

endfeet were also observed surrounding other capillaries. Specific endothelial changes such as loosening of tight junctions and increased numbers of endocytotic vesicles, however, were not evident in the examined brain sections. No abnormalities were evident in the control mouse brain sections.

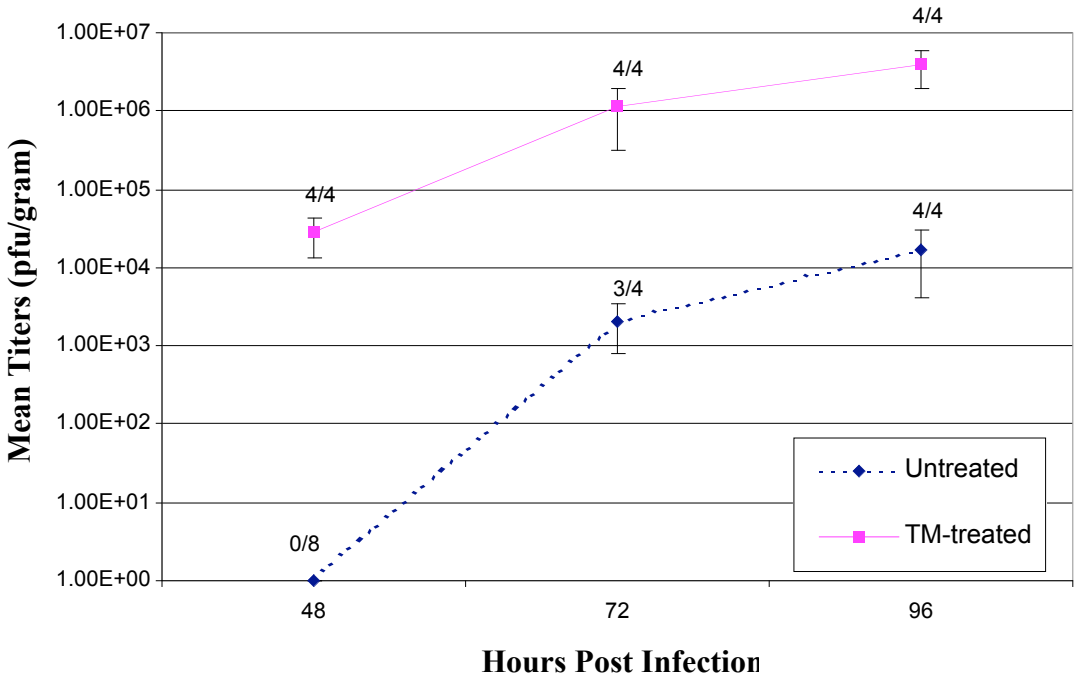



**Figure 16. Brain titers of attenuated VEE virus in tunicamycin-treated and untreated mice.** TM-treated mice infected with V3034 virus exhibit faster neuroinvasion than untreated mice. Mice were treated with TM 12 hours before inoculation of virus into the footpad and were sacrificed (4 to 8 per group) at the indicated times post infection. The fractionated numbers for each data point represent the number of positive mice divided by the number of total mice at that time. The mean brain titers of TM-treated mice were significantly greater than those of untreated mice over the timecourse of the study. The sensitivity of the plaque assay was 165 plaque forming units (pfu)/gram.

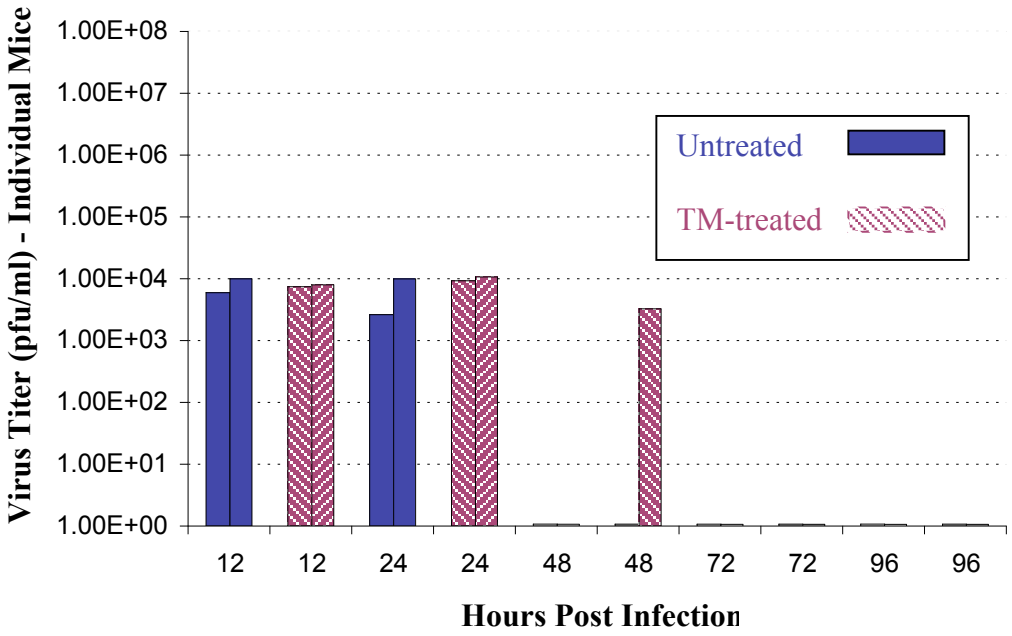



**Figure 17. Viremia with attenuated VEE virus in tunicamycin-treated and untreated mice.** Tunicamycin has no apparent effect on the development of viremia compared to untreated mice infected with V3034 virus. Mice were manipulated as described in Figure 16 and sera were processed for plaque assay. The sensitivity of the assay was 33 plaque forming units (pfu)/ml.

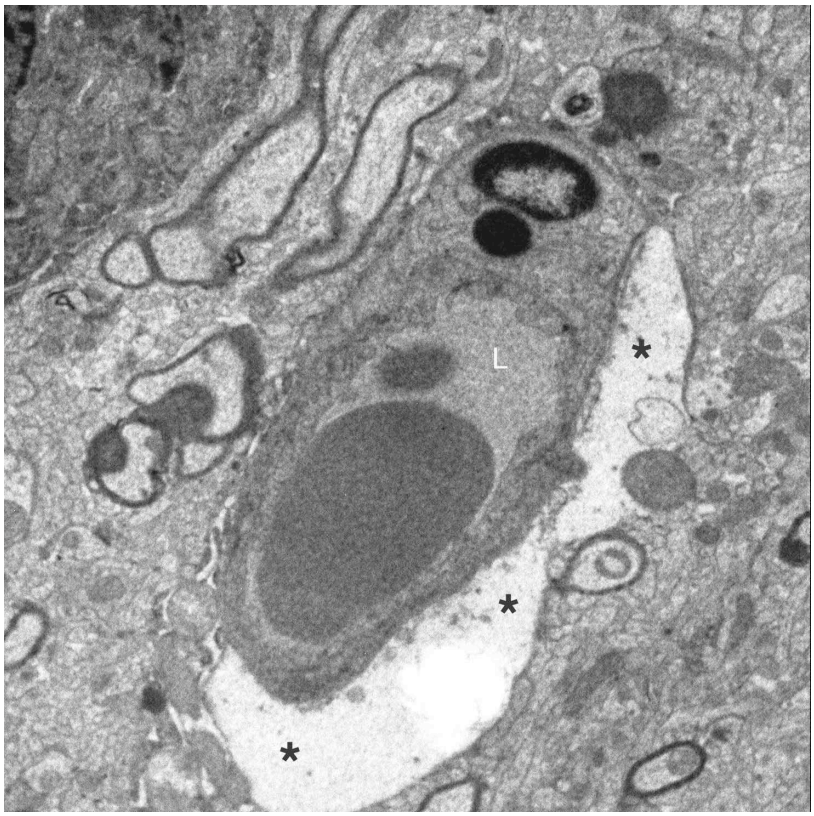



**Figure 18. Electron micrograph of a capillary in the brain of a tunicamycin-treated mouse**. Damage to the blood-brain barrier is indicated by the presence of pericapillary edema (***) in the pyriform cortex of a mouse 24 hours after treatment with 2.5 _g of tunicamycin given intraperitoneally. Within the lumen (L) of the capillary is an erythrocyte. The brain specimen was fixed in universal fixative and processed for standard transmission electron microscopy.

### D. Neuroinvasion by V3034 in Pyridostigmine and LPS-treated Mice

Neither PB nor LPS administration to mice produced statistically significant changes in the amounts of virus in the brains of mice infected with V3034 in the footpad. Some minor differences were noted however. Overall, 9 of 12 PB-treated mice demonstrated neuroinvasion from 72 to 120 hours PI, compared to 6 of 12 untreated mice (Figure 19). Considering only the latter time

Figure 19

points of this experiment, 8 of 8 PB-treated mice had neuroinvasion versus 6 of 8 untreated mice. The levels of virus reached in the brains of treated and untreated mice, though, did not differ significantly.

In the first LPS study, 2 of 6 LPS-treated mice demonstrated neuroinvasion, compared to 1 of 6 untreated mice (Figure 20). These data were

Figure 20

taken from the study designed to measure TNF-□ and NO levels that follows and the numbers of mice (and the time points) available for brain titrations from this experiment were too few to make valid conclusions. A second study was therefore conducted. In this study, overall 6 of 9 LPS-treated mice exhibited neuroinvasion, versus 4 of 12 untreated mice. At 96 hours PI, the only time point at which such a comparison is possible, the levels of virus in LPS-treated mice appear essentially the same as those in untreated mice. It is interesting to note, however, that at 72 hours PI, 2 of 3 treated mice were positive compared to 0 of 4 untreated mice.

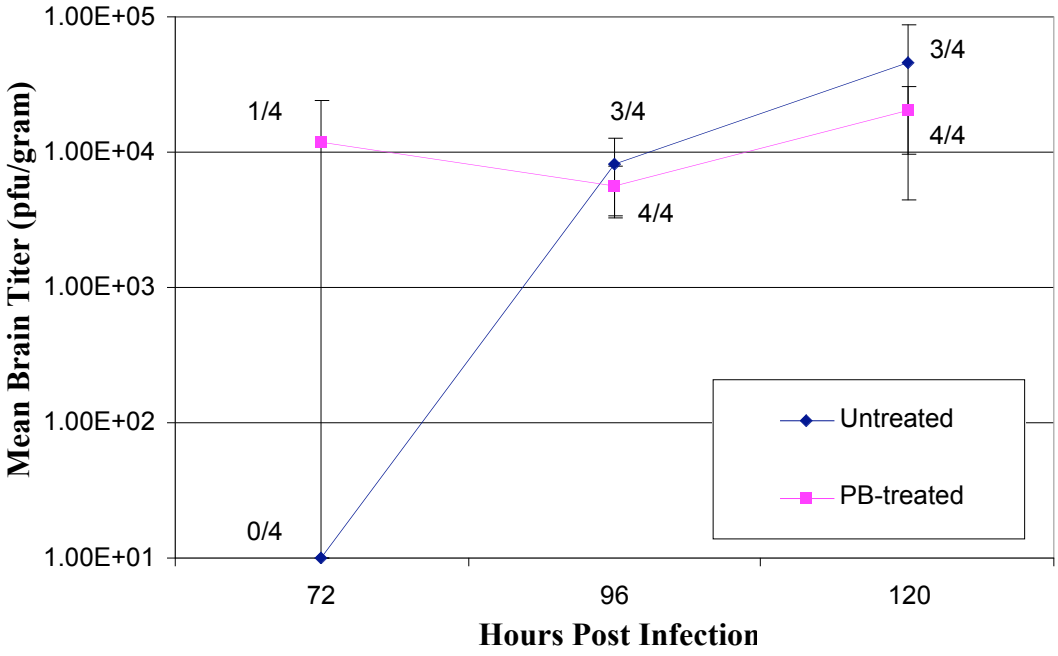
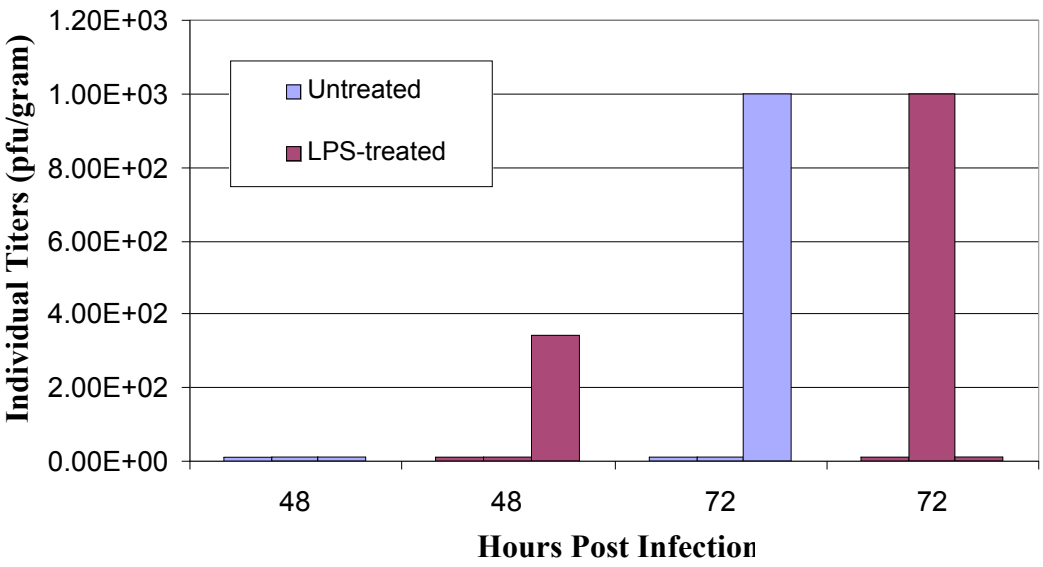


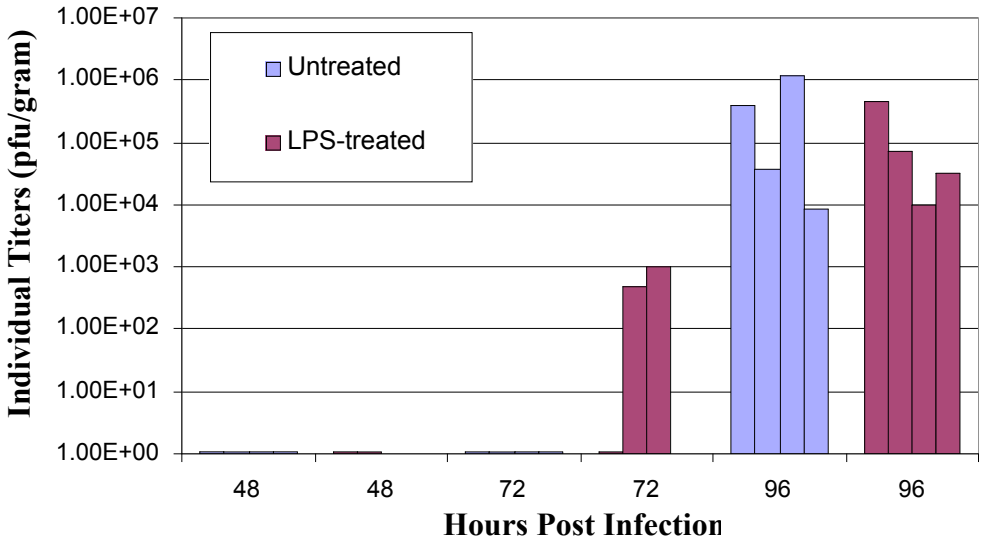

**Figure 19. Brain titers of attenuated VEE virus in pyridostigmine-treated and untreated mice.** Untreated mice and mice treated with PB show very similar neuroinvasion and early brain replication profiles after infection with attenuated VEE virus. Mice were infected with 1 x $10^3$ pfu of V3034 virus in the footpad. PB-treated mice were given 1.09 mg/kg pyridostigmine bromide by gavage at the time of expected peak viremia. Mice were sacrificed (4 per group) at the indicated times post infection and brain samples were analyzed by plaque assay. The fractionated numbers for each data point represent the number of positive mice divided by the number of total mice at that time. The sensitivity of the plaque assay was 165 plaque forming units (pfu)/gram.

**Figure 20. Brain titers of attenuated VEE virus in LPS-treated and untreated mice.** Untreated mice and mice treated with lipopolysaccharide show similar neuroinvasion and early brain replication profiles after infection with attenuated VEE virus. Mice were infected with 1 x $10^3$ pfu of V3034 virus in the footpad and were given 100 _g of lipopolysaccharide (LPS) IP in both experiments. In experiment 1, LPS was given at the time of infection. In experiment 2, LPS was given at the time of expected peak viremia. Mice were killed at the indicated times post infection and brain samples were analyzed by plaque assay. The sensitivity of the assay was 165 plaque forming units (pfu)/gram.

## Experiment 1




## Experiment 2



## E. TNF-α and NO in VEE Infection

### 1. Serum TNF-α and NO Levels in Early VEE Infection

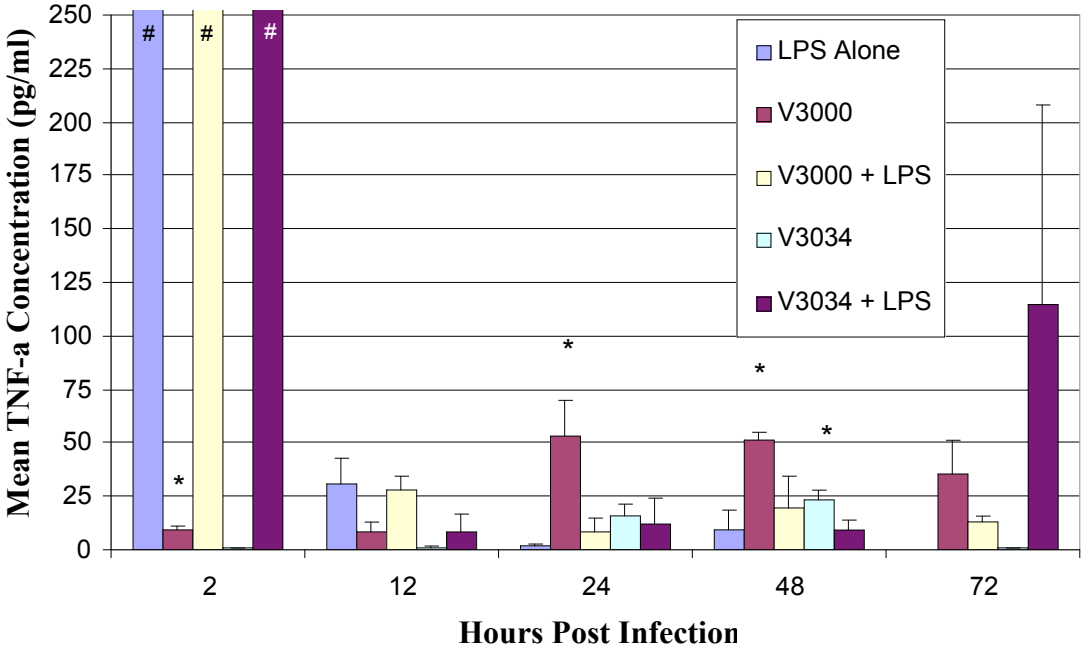
Increased serum TNF-α levels were present in mice infected with both V3000 and V3034 viruses (Figure 21). In V3000-infected mice, TNF-α levels

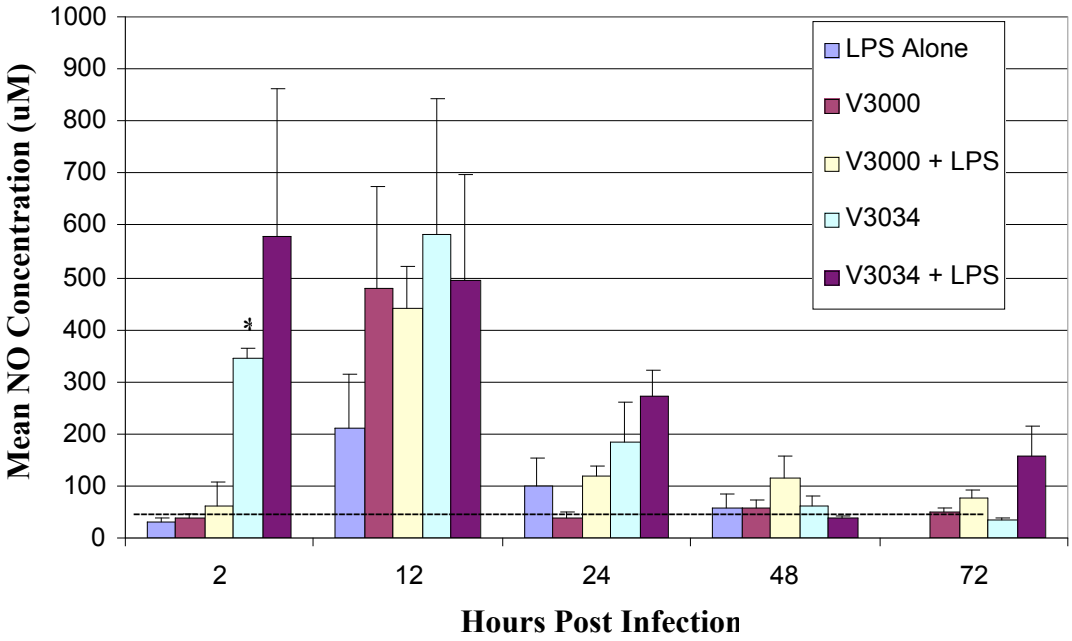
Figure 21

rose slightly at 2 and 12 hours PI, peaked at greater than 50 pg/ml at 24 hours PI, then trailed off at 48 and 72 hours PI. The differences between infected mice and uninfected mice were statistically significant at several time points. By comparison, elevations in TNF-α levels in V3034-infected mice were delayed and diminished, increasing at 24 hours PI and reaching a peak of only 23.7 pg/ml at 48 hours PI. The differences between V3034-infected and uninfected mice were significantly different only at 48 hours PI. The administration of LPS induced dramatic elevations in TNF-α levels, but this effect was only transient. In V3000 and V3034-infected mice treated with LPS and in uninfected mice treated with LPS, serum TNF-α levels of 2500 pg/ml or greater were present in all 3 groups at 2 hours PI. However, by 12 hours PI, TNF-α levels were only slightly increased in these 3 groups. In effect, LPS administration did not appear to augment serum TNF-α levels of mice infected with either virus. Interestingly, after 12 hours PI, TNF-α levels in LPS-treated, V3000-infected mice were actually lower than those in untreated mice infected with V3000. TNF-α levels were undetectable in the untreated, uninfected control mice.

Increased serum NO levels, measured as total serum nitrite, were also present in mice infected with both viruses. In V3000-infected mice, this effect

**Figure 21. Serum TNF-_ and nitric oxide levels in early VEE**. The levels of TNF-_ and nitric oxide (NO) in the sera of mice infected with V3000 and V3034 during the early course of VEE infection, with and without lipopolysaccharide (LPS) treatment, are shown. Mice were infected with either virus at 1 x $10^3$ pfu in the footpad and some groups were treated with 100 _g of LPS IP at the time of infection. Blood samples were obtained at necropsy at the indicated times. TNF-_ levels were measured by ELISA and NO levels (as total nitrite) were measured by the Griess reaction. The levels of TNF-_ in untreated/infected mice were significantly different ($p < .05$) than 3 uninfected/untreated control mice (which had undetectable levels of TNF-_) as indicated (*). The levels of NO in untreated/infected mice were significantly different ($p < .05$) than uninfected/untreated control mice (dashed line indicates the mean value of NO concentration for 3 negative control mice (76.5 +/- 28.1 uM)) only for V3034-infected mice at 2 hours PI (*). All groups of LPS-treated mice showed transient, but dramatic spikes in TNF-_ levels off the chart. The absolute values (+/- SEM) for these 3 data points (#) are, in order, 2672 (+/- 1299.9), 2505.8 (+/- 685.1), 2848.7 (+/- 649.9) pg/ml).


Mean TNF-a Concentration (pg/ml)
250
225
200
175
150
125
100
75
50
25
0
#
#
#
*
*
*
*
LPS Alone
V3000
V3000 + LPS
V3034
V3034 + LPS
2
12
24
48
72
Hours Post Infection


Mean NO Concentration (uM)
1000
900
800
700
600
500
400
300
200
100
0
*
LPS Alone
V3000
V3000 + LPS
V3034
V3034 + LPS
2
12
24
48
72
Hours Post Infection

was very transient, as serum nitrite was elevated above baseline only at 12 hours PI. These differences were not significantly different, however. In V3034-infected mice, increased serum NO was seen at 2 hours PI, however, and peaked at 12 hours PI before trailing off to about baseline level by 48 hours PI. The differences between V3034-infected and uninfected mice were significantly different only at 2 hours PI. Treatment of mice with LPS also resulted in early, transient elevation of NO levels, somewhat similar to its effect on TNF-□. However, LPS did not appear to significantly alter the NO levels in the sera of mice infected with either V3000 or V3034 compared to untreated mice.

**2. Neuroinvasion by V3000 in Mice Treated with Antibodies to TNF-□**

Treatment of mice with antibodies to TNF-□ had no apparent effect on neuroinvasion by V3000. Neuroinvasion was undetectable in mice of treated and untreated groups at day 1 PI (Figure 22). At days 2, 3 and 5 PI, both groups of mice had steadily increasing mean brain titers that were nearly identical.

Figure 22

**F. The Effect of TM on VEE Virus Replication *in vitro***

To begin to understand the effect of TM on VEE virus replication, an *in vitro* study was performed in which V3000 replication was compared in TM-treated versus untreated BHK-21 cells. These cells represent a permissive, nonneuronal (fibroblast) cell line. In this experiment, the untreated control cells demonstrated the expected virus replication curve (Figure 23). V3000 replicated

Figure 23

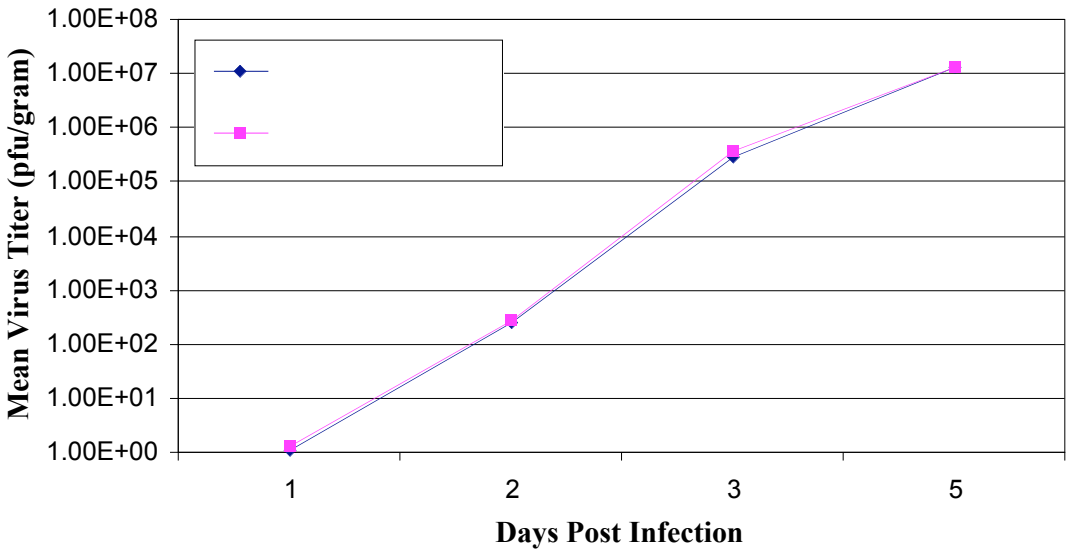



**Figure 22. Neuroinvasion in the brains of TNF-_ antibody-treated and untreated mice infected with virulent VEE virus**. Untreated mice and mice treated with antibodies to TNF-_ show nearly identical neuroinvasion and early brain replication profiles after infection with virulent VEE virus. Mice were infected with 1 x $10^3$ pfu of V3000 in the footpad and antibody-treated mice received 2 _g of goat polyclonal antibodies to mouse TNF-_ subcutaneously at the time of infection. Mice in the 5 day group received a second antibody treatment at 72 hours PI. Mice were killed, 6 per group, at the indicated times and the brains of all mice were processed for virus titration by plaque assay.

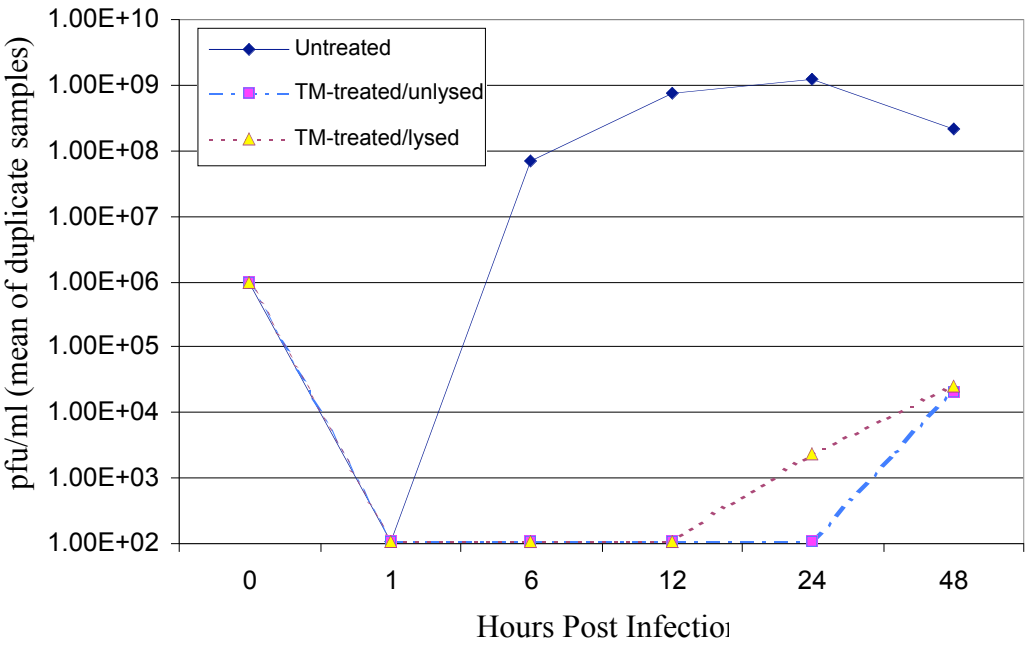



**Figure 23. Replication of virulent VEE virus in tunicamycin-treated and untreated BHK-21 cells**. VEE virus shows delayed and diminished replication in TM-treated BHK-21 cells compared to untreated cells. Tunicamycin treatment was with 0.5 _g/ml for 12 hours prior to infection with V3000 at a MOI of 1.0. At the indicated times, culture medium was sampled (unlysed), plates were processed through 3 freeze thaw cycles to lyse cells and the remaining medium was sampled (lysed). All samples were titered by plaque assay.

rapidly to nearly 1 x $10^8$ pfu/ml by 6 hours PI, peaked at more than 1 x $10^9$ pfu/ml at 24 hours PI, then declined. In contrast, TM-treated cells showed a much delayed and diminished replication pattern. V3000 was undetectable in the medium at 12 hours PI in both lysed and unlysed samples. The lysing procedure was included to ensure release of virus that may have been unable to exit cells due to the TM treatment. Medium from the lysed samples did contain, on average, more than 1 x $10^3$ pfu/ml at 24 hours and more than 1 x $10^4$ pfu/ml by 48 hours, when the experiment was terminated. Medium from the unlysed samples had no detectable virus at 24 hours PI, but contained more than 1 x $10^4$ pfu/ml at the 48 hour mark.

Immunostaining for infected BHK-21 cells in 8-well chamber slides was performed to complement the virus titer data. At 1, 6 and 12 hours PI, the mean numbers of antigen-positive cells per well in the untreated samples were 27.5, 737.5 and 2232 respectively. In the TM-treated samples, the mean numbers of positive cells per well were 32.5, 347 and 763 respectively. Data were not obtained for the 24 and 48 hour samples, as many of the cells in the TM-treated samples were dead.

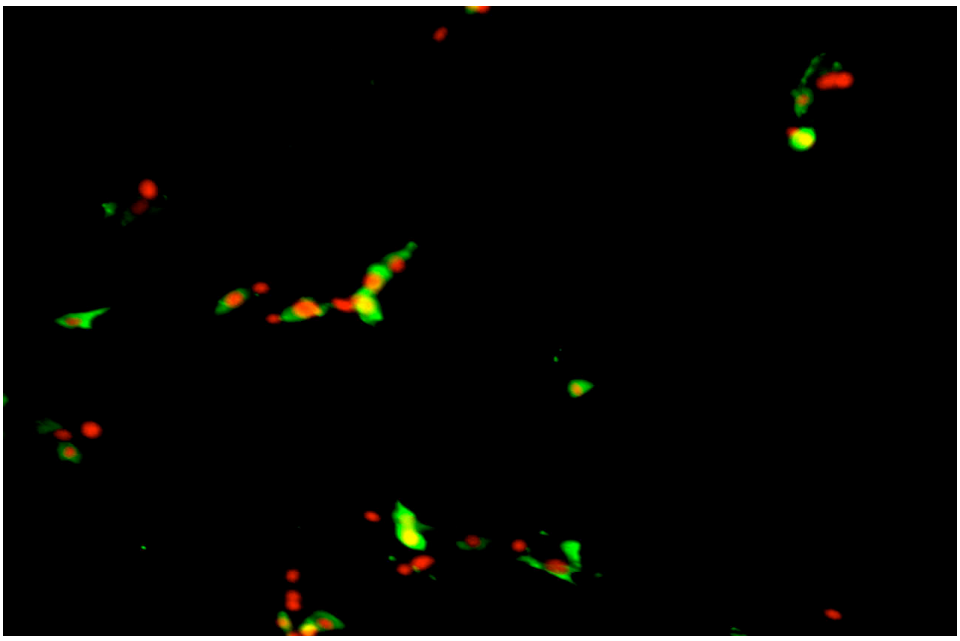
Some differences were observed in the morphologic features of untreated and TM-treated cells that contained VEE virus antigen. From 1-6 hours PI, antigen in the untreated cells was generally perinuclear to diffusely cytoplasmic. In some cells, the immunostaining was finely granular. At later times, the antigen tended to be concentrated along the plasma membrane of cells. The morphology

of antigen-positive cells remained relatively normal, i.e. cells were generally compact and spindled (Figure 24A) until 48 hours PI. In TM-treated cells,

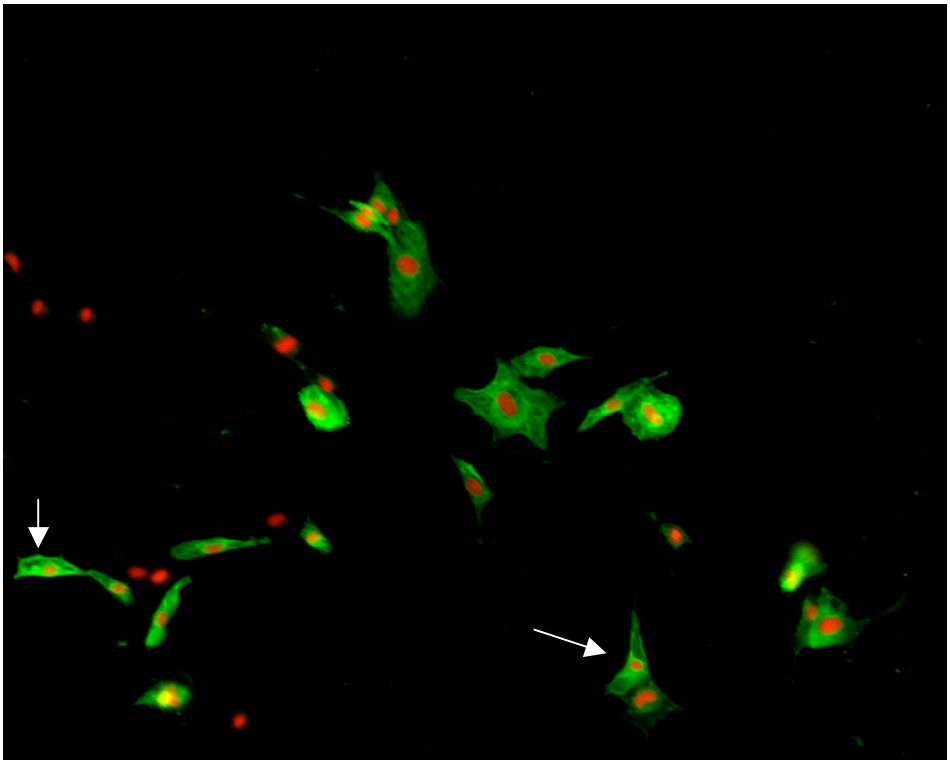
Figure 24

antigen-positive cells at 1 hour PI were similar to the untreated cells. At 6 hours and later, though, antigen-positive cells often contained large cytoplasmic vacuoles from which virus antigen was excluded. Fewer cells contained granular antigen, as seen in some untreated cells. TM-treated cells that were antigen-positive at 6 hours PI and later were often misshapen (Figure 24B), taking on a more polygonal to rounded appearance. No obviously abnormal cytological features were noted in the TM-treated cells that did not contain antigen.

**Figure 24. VEE virus antigen expression in untreated and tunicamycin-treated BHK-21 cells**. In untreated BHK cells (A), infected cells (bright green) generally maintain their compact, spindled appearance and virus antigen is present throughout the cytoplasm. In TM-treated cells (B), infected cells are often enlarged and rounded to polygonal and have cytoplasmic vesicles (arrows) devoid of virus antigen. Cells were infected with V3000 at a multiplicity of infection (MOI) of 1.0. TM treatment was 0.5 ug/ml TM for 12 hours prior to infection. In both cases, slides were fixed in 10% neutral-buffered formalin 24 hours PI and stained for VEE antigen using a polyclonal antibody; propidium iodide (red) was used as a nuclear counterstain.



A



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# IV. DISCUSSION

The overall aim of this research was to investigate whether specific chemical and host factors could alter the pathogenesis of Venezuelan equine encephalitis (VEE) virus in mice and to attempt to elucidate the mechanisms by which they might do so. In particular, tunicamycin (TM), a chemical shown previously by this laboratory to enhance the pathogenicity of the related alphavirus Semliki Forest virus (SFV) and encephalomyocarditis virus (EMCV),[41] was the major focus of this work. Results from the early portions of this research indicated that TM enhanced the pathogenicity of virulent VEE virus in mice and that enhanced neuroinvasion appeared to be an important component of this effect. Subsequent experiments were performed to further investigate the effect of TM on the process of neuroinvasion.

One of the findings of these studies provided support for previous research indicating that TM caused damage to the blood-brain barrier (BBB).[70] Because the notion of alteration of the BBB by TM provided an appealing explanation for enhancing the pathogenicity of VEE virus, it was later decided to explore the possibility that other agents known to alter the BBB, pyridostigmine bromide (PB), lipopolysaccharide (LPS) and TNF-_, might also prove to enhance neuroinvasion by VEE virus. In fact, as discussed later, the data obtained from these studies failed to show that these agents enhanced VEE virus neuroinvasion comparable to TM. These results imply that TM might be relatively unique in its effect on the pathogenesis of VEE and could suggest a possible mechanism by which TM acts with respect to VEE, as further discussed later. The findings of

this research should provide direction for future studies to help explain the process of neuroinvasion in VEE. Understanding how this process occurs could lead to means by which neuroinvasion by VEE virus or other encephalitic viruses could be inhibited, thus limiting or preventing the damage caused by these important diseases.

## A. Agents that Alter the Blood-brain Barrier and their Effects on VEE

### 1. Tunicamycin and Virulent VEE Virus: Viremia, Neuroinvasion and Intracranial Replication Profiles

The mortality study with virulent VEE virus showed that TM caused a significant decrease in the mean survival time (MST) of mice compared to untreated mice. The MST is the key feature of this study because both groups exhibited 100% mortality, as is typical of virulent VEE virus in mice.[25, 26, 38, 43] A followup experiment designed to broadly characterize the disease in mice treated with TM provided two important findings with respect to a possible mechanism by which TM affects VEE. First, TM did not appear to alter the production of viremia, as untreated and treated mice had very similar viremia curves, also typical of virulent VEE.[26, 30] Second, TM did significantly increase the levels of V3000 in the brain early in the course of infection. In particular, TM-treated mice had much greater virus titers in the brain at 48 hours post inoculation (PI) than untreated mice. This seems the key time point at which to compare the two groups. At earlier times, the process of neuroinvasion, which requires that viremia first occur, probably had insufficient time to develop, even with the benefit of TM treatment. At later times, neuroinvasion by virulent VEE virus

occurs effectively even without TM treatment. It is therefore not too surprising that statistically significant differences in brain virus titers between groups of TM-treated and untreated mice were only seen at 48 hours PI. These results suggested that TM enhanced neuroinvasion by virulent VEE virus.

It was necessary, though, to rule out the alternate possibility that virulent virus simply replicated much faster in the brains of mice under the influence of TM. In the experiment in which TM-treated and untreated mice were inoculated with V3000 intracranially, there was no difference in virus replication in the brain. This finding was considered important and further strengthened the belief that TM enhanced neuroinvasion.

**2. Tunicamycin and Attenuated VEE Virus: Focus on Neuroinvasion**

Because virulent VEE virus is 100% neuroinvasive in mice, work with the attenuated VEE virus strain V3034 provided an alternative to studying neuroinvasion as it has previously been shown to be less efficient than V3000 in invading the brains of CD-1 mice,[26] the mouse strain used for the current studies. The data obtained using V3034 extended the findings with virulent VEE virus and were particularly notable. Tunicamycin treatment resulted in neuroinvasion in all of the mice from 48 hours onward. Tunicamycin treatment also caused much higher virus titers in the brain versus untreated mice at all time points. As with V3000, TM did not alter the viremia curve in mice infected with V3034. This study, together with the preceding ones, supports the conclusion that TM affects the process of neuroinvasion.

### 3. Tunicamycin and the Blood-brain Barrier: Consequence for Neuroinvasion

Electron microscopy was used to show that TM caused some damage to the BBB. This damage was relatively minor, however, as the only changes evident ultrastructurally were mild edema surrounding vessels and some swollen astrocytic endfeet. These observations are consistent with the earlier report that TM-induced damage to the BBB of guinea pigs was relatively subtle.[70] The demonstration of TM-induced damage to the BBB provided a possible explanation for the means by which TM enhanced neuroinvasion, i.e. that VEE virus was able to cross a damaged BBB and directly invade the brain.

The immunohistochemistry findings from the pathogenesis experiment with V3000 failed to support this idea. In general, virus infection via the BBB is expected to exhibit two key features: first, that the virus appears in multiple regions of the brain at essentially the same time and second, that the virus appears in a perivascular pattern. In addition, endothelial infection may be apparent. In contrast to these features, the immunohistochemistry findings indicated that V3000 appeared first in the olfactory-related portions of the brain in both TM-treated and untreated mice, there was no perivascular pattern of virus antigen in both experimental groups and there was no evidence of endothelial infection. VEE virus did not appear in caudal regions of the brain until later. Based on these findings, virus neuroinvasion in TM-treated mice appeared to follow the model already established for VEE virus and several other arboviruses.[43, 58, 59, 60] This model contends that some encephalitis viruses escape from the blood in the region of the olfactory tract and either infect olfactory neurons or simply invade their cell

processes (axons) which form the olfactory nerves. These viruses then ascend into the brain, appearing initially in the olfactory bulbs and subsequently spreading to other parts of the brain. Taken together, the findings of the current work indicate that TM does cause damage to the BBB, but that this damage is inconsequential to neuroinvasion by VEE virus.

**4. Lipopolysaccharide and Pyridostigmine and their Effects on VEE**

The studies performed with other agents known to damage the BBB appear to support this latter assertion as neither LPS nor PB caused significant alterations to mortality or neuroinvasion with VEE viruses. The effect of PB on mortality due to virulent VEE virus included 3 separate experiments in which the timing of PB administration was altered. In all 3 experiments, the MST of the PB-treated and untreated groups were not significantly different. Likewise, LPS failed to significantly alter the MST of mice infected with virulent VEE virus. In addition, an experiment with the attenuated strain V3010 similarly failed to show a significant alteration of mortality by PB. V3010 was chosen for this study rather than V3034 because a previous report indicated that V3010 was more neurovirulent,[26] which suggested that any increase in neuroinvasiveness due to PB was more likely to be manifest as increased mortality using V3010 than V3034. It should be noted that a single mortality in the PB-treated group perhaps suggests some effect from PB that might take on statistical significance in an experiment involving a much larger group of mice.

Nonetheless, these studies with LPS and PB failed to show any significant enhancement of mortality by VEE viruses. Direct evidence of BBB damage by

LPS or PB was not provided by the current studies. However, the experiments with SFV and EMCV did show that PB treatment increased mortality to 50% compared to 0% in untreated mice infected with both of these viruses. These latter findings are important in that they show, at least for PB, that an agent known to alter the BBB enhances the pathogenicity of encephalitic viruses other than VEE virus. They also extend the findings published previously showing that TM increased mortality in mice infected with SFV and EMCV.[41]

The neuroinvasion studies with LPS and PB supported the mortality studies involving these agents. They showed that both LPS and PB failed to produce significant differences in the numbers of mice exhibiting neuroinvasion with the attenuated strain V3034. Actually, there were slightly more mice exhibiting neuroinvasion with V3034 in both LPS-treated and PB-treated groups versus untreated groups suggesting some underlying effect due to chemical treatment. Even so, these increases were unimpressive compared to the prominent increase in neuroinvasion in TM-treated mice infected with V3034. Again in contrast to the VEE studies, a neuroinvasion experiment in this laboratory involving SFV showed higher mean titers in the brains of mice treated with PB than in untreated mice, especially at early time points (P. Seth, unpublished data). This work supports the SFV mortality study with PB and is consistent with studies by others showing that PB enhanced neuroinvasion due to Sindbis virus[71] and LPS enhanced neuroinvasion and mortality in mice infected with West Nile virus and Sindbis virus.[64]

The failure of the current studies to show that LPS and PB could enhance neuroinvasion and mortality due to VEE viruses appears to support the previous assertion that damage to the BBB per se is not of great consequence to the pathogenesis of VEE. That these same agents, as well as TM, are able to enhance the pathogenicity of other encephalitic viruses should not be too surprising given the general notion that some viruses, such as SFV, invade the brain via the BBB while others, including VEE virus, utilize the olfactory system. In this regard, it would be interesting to investigate whether agents like LPS and PB might also fail to alter the pathogenicity of other viruses that use the olfactory route of neuroinvasion, like St. Louis encephalitis virus, louping ill virus and yellow fever virus. It would also be interesting to show that TM could enhance neuroinvasion by these viruses.

**5. The Possible Role of TNF-_ and Nitric Oxide in VEE**

The studies involving TNF-_ and NO were done to provide a basis for determining if host factors themselves may play a role in facilitating neuroinvasion. TNF-_ and NO were specifically chosen because previous studies have indicated that they can alter the BBB.[65, 66, 67, 68] The findings here that serum TNF-_ and NO were both upregulated during the neuroinvasive phase of VEE in mice suggests that these factors may play a contributory role in neuroinvasion with VEE virus. In that regard, several points from this study are of interest. First, serum TNF-_ reached higher levels after infection with the more neuroinvasive V3000 virus than with V3034. In addition, serum TNF-_ peaked at 24 hours pi after V3000 infection and at 48 hours pi after V3034 infection. This

may be interesting in light of the facts that, in the current studies and in other reports,[26, 43] neuroinvasion is accomplished earlier with V3000 (24-48 hours pi) than with V3034 (72 hours pi). In contrast to TNF-_, serum NO reached higher levels after infection by V3034 than by V3000. Also, there was no correlation apparent between the timing of peak NO concentrations and the timing of neuroinvasion due to these viruses. These findings suggested that TNF-_ was a more likely candidate to play a role in alteration of the BBB and facilitate neuroinvasion during VEE than was NO.

This possibility, however, was not supported by the experiment involving treatment of mice with antibodies to TNF-_, as antibody-treated mice did not exhibit inhibition or delay of neuroinvasion by virulent VEE virus compared to untreated mice. This result is probably not surprising, given the relatively modest serum levels of TNF-_ seen in VEE-infected mice. Actually, the TNF-_ levels seen were well below those reported in a recent study of VEE virus infection in mice.[81] There were several differences, however, that make comparing the two studies difficult. These include the use of different strains of virulent VEE virus, different strains of mice and the exact means of measuring serum TNF-_. The doses of viruses used ($1 \times 10^3$ pfu in the current studies versus $6.8 \times 10^7$ pfu in the previous report) and the time points considered also were different.

The finding of higher NO levels in V3034-infected mice compared to V3000-infected mice could conceivably have other significance. V3034 is much less virulent than V3000. V3034-infected mice achieve lower levels of viremia, decreased neuroinvasion and limited mortality after infection by either the

peripheral or intracranial routes. It is interesting to speculate that higher NO levels in V3034-infected mice may have some protective function, based on a number of previous studies that have attributed antiviral abilities to NO.[82, 83, 84, 85, 86, 87] This possibility requires further study. It should be mentioned that NO expression within the brain has actually been associated with increased pathogenicity in VEE.[37] It is difficult to make a connection between that study and the current one, however, as the current study investigated early NO changes in the blood, versus later changes in the brain.

The data regarding the effect of LPS administration on serum TNF-_ also deserve some mention. There was a dramatic but short-lived increase in serum TNF-_ levels in mice treated with LPS. It could be argued that this short-lived effect might explain why administration of LPS to mice at the time of infection with V3000 in the mortality study did not alter the course of disease with respect to MST. In effect, the LPS-induced elevation of TNF-_ in infected mice occurred prior to the neuroinvasive phase of VEE. The results of the neuroinvasion study with V3034 contradicts this premise, however, as there was no significant enhancement of neuroinvasion by V3034 in mice treated with LPS at peak viremia.

**B. <u>The Effect of Tunicamycin on Encephalitis</u>**

In addition to enhancing neuroinvasion by VEE viruses, TM treatment of mice also resulted in increased neurodegenerative changes during the encephalitic phase of VEE. The brains of TM-treated mice had greater neuronal damage and inflammatory infiltrates at equivalent time points than did untreated mice. TM-

treated mice also had increased fibrinogen leakage from blood vessels in the brain and greater upregulation of some key cytokines at equivalent time points than did untreated mice. In general, these changes began to appear about 24 hours after documented infection of the brain. Qualitatively, the neurodegenerative changes in TM-treated mice did not appear different than those in untreated mice. In other words, more severe encephalitic changes in TM-treated mice correlated with more virus in the brain at early time points, indicating that the enhanced neurodegenerative changes in the brain subsequent to TM treatment were mostly secondary to the earlier neuroinvasion.

It is necessary to point out, however, that previous reports showed that neurons are subject to the toxic effects of TM *in vitro*, and are affected *in vivo* by natural poisoning with the related toxin (corynetoxin).[70, 88, 89] This raises the important possibility that TM-related neurotoxicity could have contributed to the greater neurodegeneration that resulted from virus infection of the brain. A lethal dose of TM in mice was previously determined by this laboratory to be 10 _g/mouse or greater.[41] In the current studies using a TM dose of 2.5 _g per mouse, neither clinical nor histopathological abnormalities were evident in uninfected mice treated with TM, with the exception that they failed to gain weight as rapidly as untreated control mice. In particular, neurons in the brains of TM-treated, uninfected mice exhibited no morphological evidence of TM-induced neurotoxicity.

Despite the conclusion that the enhancement of neurodegenerative changes in the brains of TM-treated mice was probably in large part secondary to

enhanced neuroinvasion and even with evidence of neurotoxicity in uninfected mice given the same dose of TM lacking, it remains possible that TM-induced changes may have contributed to the severity of encephalitis resulting from VEE virus infection. For example, TM could have induced neuronal changes that did not result in morphologic changes but functionally altered neurons and combined with virus infection, contributed to neurovirulence. Another possibility is that TM-induced changes in the BBB may have contributed to the inflammatory response to virus infection. Alterations to the BBB can permit increased leakage of soluble blood elements and facilitate infiltration of inflammatory cells into the brain. As an example, increased expression of the adhesion molecule _-4 integrin on cerebral vessels of mice has been shown to facilitate inflammatory cell infiltration in the brains of mice infected with SFV.[90] Tunicamycin has the potential to alter the functionality of numerous cell surface glycoproteins such as adhesion molecules, as many of them contain N-linked sugar modifications. In the current studies, both increased fibrinogen leakage and more severe inflammatory infiltrates were present in TM-treated mice than in untreated mice. Both of these phenomena have been associated with neurodegeneration via immunopathologic mechanisms in a variety of CNS disorders.[76, 90, 91] Thus, BBB alterations induced by TM could have worsened the immunopathologic damage to the brain which has been reported to occur in VEE.[92] This possibility should be a focus of future studies, in which the timing of TM administration would be important. In effect, reserving TM treatment until after the neuroinvasion phase is mostly passed and the encephalitic phase is in process and then comparing

encephalitic changes in treated and untreated mice could demonstrate whether TM has such an effect.

### C. Ancillary Findings

Some additional observations made in the course of the current studies deserve discussion. First, as expected, neurons were the primary target of VEE virus infection in the brains of both TM-treated and untreated mice. This observation was provided by double labeling for virus antigen and a neuronal marker. Unexpectedly, though, double labeling for virus antigen and glial fibrillary acidic protein (GFAP) showed an almost complete lack of apparent astrocyte infection. This is in contrast to a previous study that showed that astrocytes were susceptible to VEE virus infection *in vitro*.[32] The disparity in these 2 studies may simply indicate differences in astrocyte susceptibility to VEE virus infection *in vivo* versus *in vitro*. Alternatively, it is conceivable that GFAP labeling failed to identify some infected astrocytes in the current study. GFAP expression in gray matter astrocytes is generally weaker than that in white matter astrocytes. VEE virus infected cells were observed primarily in the gray matter of the brain. In addition, while GFAP expression is generally stronger in "activated" astrocytes, it is not known what effect VEE virus infection might have on GFAP expression in astrocytes, should it occur. In other words, downregulation of GFAP in VEE virus-infected astrocytes could preclude their identification using GFAP-based immunohistochemistry. Additional work using other astrocyte markers is probably required to sort out this potential problem.

One characteristic of the encephalitis that deserves mention is the type of neuronal damage seen in both TM-treated and untreated mice with VEE. Neuronal apoptosis has been reported to occur as a consequence of infection by VEE virus and other alphaviruses.[38, 50, 52] Other studies, though, characterize the neuronal damage in VEE as the result of necrosis.[28, 42] In the current studies, neurons with morphologic features of apoptosis were observed in the brains of both TM-treated and untreated mice with VEE. Apoptotic changes were particularly prominent in hippocampal neurons and cerebellar granule neurons. It is interesting that apoptosis of neurons in these two particular neuronal cell types has recently been associated with alterations of neurotrophin and neurotrophin receptor levels in rats with Borna disease.[93] Further of interest, TM has been shown to inhibit neurotrophin-receptor binding [94] and to induce apoptosis of cerebellar granule cells.[95] It is therefore tempting to speculate that TM may add to the neuronal apoptosis of cells already under assault by VEE virus. In addition to apoptotic neurons, damaged neurons with morphologic features of necrosis were present in other parts of the brain. These were especially common in the pyramidal layer of neurons of the pyriform cortex as well as other populations of large neurons. As with apoptotic neurons, necrotic neurons were also seen in both groups of mice with VEE.

Surprisingly, neurons with morphological features of both apoptosis and necrosis were labeled by the TUNEL method. It was further surprising that most labeled neurons, also including those with features of apoptosis and necrosis, exhibited primarily cytoplasmic staining and lacked the nuclear staining

characteristic of apoptosis. The reason for these unusual findings is uncertain. While these results may not have great relevance to the subject of TM-induced neurovirulence, it is worth pointing out that VEE virus itself appears to result in damage to neurons by both apoptosis and necrosis and that the outcome of neuronal infection may be related to the type of neuron involved.

The *in vitro* study may offer some insight into the effect of TM on VEE virus interaction with susceptible cells and will be mentioned briefly. This limited study indicated that VEE virus inoculation of BHK-21 cells treated with TM prior to infection resulted in limited and delayed virus replication. This result was obtained using a methodology similar to one used in recent studies investigating the effect of host cell receptor protein glycosylation on infectivity by retroviruses.[96, 97] However, it is unclear if the reduced VEE virus replication in TM-treated BHK-21 cells was the result of inefficient virus infectivity (presumably due to altered glycosylation of a host cell receptor protein) or the effect of TM on subsequent virus production (presumably due to altered virus protein glycosylation). Importantly, the cited studies also employed the methodology of virus transduction to directly show that TM-induced alteration of receptor protein glycosylation specifically affected the process of viral infectivity of host cells. The recent studies also employed other methods not used in the current *in vitro* study. In short, because the *in vitro* study presented here was of such limited scope, the results should be interpreted with caution. Additional studies are necessary to more specifically determine the effect of TM on VEE virus interaction with susceptible cells.

## D. A Proposed Concept of How Tunicamycin Alters the Pathogenesis of VEE

Considering the data provided by the current studies in light of relevant previous reports, the following concept concerning the action of TM begins to emerge. In effect, chemical agents (TM, PB and LPS) that alter the BBB appear to enhance neuroinvasion, and mortality, by viruses that cross vessels in the brain (particularly SFV). TM, though, appears relatively unique among these agents in its ability to also enhance neuroinvasion by VEE virus that utilizes the olfactory route. If such a proposition is true, it begs the question by what means does TM enhance VEE virus neuroinvasion if not through its effect on the BBB. This question should be the subject of future studies.

One possible explanation for how TM could enhance neuroinvasion involves altered glycosylation of host or viral proteins. This possibility is based on the following information. Many cell-surface glycoproteins including cell adhesion molecules, their ligands or other molecules contain N-linked glycosylation sites.[98, 99, 100, 101, 102] They are therefore susceptible to alteration by TM. The reports just cited concerning TM-induced alteration of retrovirus receptors is especially relevant to this discussion, as they illustrate how TM can specifically affect the interaction between viruses and host cells. Further, some N-linked glycoproteins are expressed at sites of potential importance to the process of neuroinvasion in VEE, such as on endothelial cells [100] and on axons.[103] Endothelial cell adhesion molecules such as ICAM-1 and VCAM-1 in particular are known to be involved in virus-host interactions for a variety of viruses, including HIV-1, HTLV-1, SFV and rhinoviruses.[54, 56, 57, 104] Conceptually, then,

the possibility that TM could alter glycoproteins that are involved in VEE virus-host interactions provides an interesting scenario.

While much about the mechanisms of neuroinvasion in VEE remain to be discovered, it appears that they involve important processes that occur in the olfactory tract. In one important study, it was shown that VEE virus was detectable surrounding vessels in the lamina propria of the olfactory tract at about the time of peak viremia and before virus appeared in the brain.[43] Although there was no direct evidence provided to support it, this report concluded that VEE virus probably exited the vasculature via fenestrated capillaries that supply the olfactory tract and may have invaded the peripheral nervous system through the unmyelinated axons (or the olfactory neuronal bodies from which they arise) that also lie in this region. This conclusion highlights two key events in the process of neuroinvasion: virus exit from the blood stream and virus entry into the olfactory nerves. The host cellular components involved in these events could express glycoproteins with which VEE virus interacts and that could be altered by TM. Alternatively, TM could alter viral glycoproteins (E1 and E2) that might change the interaction between virus and host cell proteins.

It is therefore tempting to speculate that TM alters either host cell or viral proteins and that this facilitates the interaction between virus and host cells in a way that enhances neuroinvasion. Altered host glycoproteins could be present on the surface of endothelial cells lining olfactory vessels, on the surface of olfactory neurons or their axons, or both. Unfortunately, there is little, if any, direct evidence to support such a concept. In fact, the receptor for VEE virus has never

been identified and there are no reports of VEE virus interaction specifically with the surface of endothelial cells, olfactory neurons or neuritic processes. These are areas which clearly require further study.

Given the very speculative nature of the preceding discussion, it is necessary to consider other possible mechanisms to explain how TM could enhance VEE virus neuroinvasion. One possibility is that altered glycoproteins involved in the host immune response could be involved. Another involves the potential for TM to alter neuronal and axonal function, as previously discussed. It is not immediately evident, however, in what ways these would affect the process of neuroinvasion, which the current studies show is at the center of the TM effect. Perhaps a more simple explanation is that TM-induced changes to vessels in the brain, as documented here and previously, are accompanied by similar changes to vessels in the olfactory tract and that VEE virus, for whatever reason, prefers the olfactory route.

In the place of further speculation about the means by which TM might alter the process of neuroinvasion in VEE, this section will be concluded with two statements for which there is some scientific basis. First, TM has the potential to alter viral glycoproteins and myriad host glycoproteins. It seems impossible to predict based on current knowledge which, if any, might be involved in TM-induced enhancement of VEE. Second, there is little fundamental understanding of the specific mechanisms by which VEE virus invades the brain, in particular how virus exits the vasculature and how it enters the nervous system. A systematic analysis of this process, focusing on blood vessels and olfactory

neurons and axons during the neuroinvasion phase of VEE, is required before further speculation about TM is appropriate.

**E. Possible Biological Relevance of These Studies**

The current studies confirm that TM should be added to the growing list of chemical agents and other factors capable of enhancing several viral encephalitides. This list currently includes LPS, acetylcholinesterase inhibitors, TNF-_ and cocaine,[55, 61, 64, 71] as well as antimalarial drugs such as chloroquine.[106] Although TM was utilized as an experimental tool based on this laboratory's previous work with the toxin and other encephalitic viruses,[41] the finding that TM increases neuroinvasion and neurovirulence in mice experimentally infected with VEE viruses may have some biological relevance. The tunicamycin antibiotics are very closely related to the toxin (corynetoxin) produced by the bacterium *Clavibacter toxicus*. Corynetoxin contaminates several plant species, including annual ryegrass, on which *C.* toxicus grows. Ingestion of the toxin by livestock grazing on contaminated plants results in the neurologic disease known as annual ryegrass staggers (ARGT).[70, 105] Tunicamycin is reported to be biologically indistinguishable from corynetoxin and is therefore often used experimentally to study ARGT.[89] The current findings together with previous studies indicate that animals which ingest corynetoxin or similar toxins in sufficient quantities could have increased susceptibility to viral encephalitis.

The finding that an attenuated strain of VEE virus was made more neuroinvasive by TM also may have biological relevance. This finding supports previous studies,[64, 71] and combined they demonstrate that viruses with limited

neuroinvasive abilities can become more neuroinvasive when host factors such as barriers to neuroinvasion are altered. Such findings could be relevant to understanding why certain individuals such as the young and the elderly are particularly disposed to develop encephalitis in the course of infection by a number of viruses. Also, with regard to future studies of neuroinvasion, infection of mice with attenuated strains may provide a more realistic model of VEE virus infection in humans, which involves infection of the brain in a minority of cases. This is in contrast to the mouse model of VEE with virulent virus, which involves neuroinvasion in 100% of infected mice.[26, 28, 42]

Much work remains to be done to identify specific mechanisms by which VEE virus and other encephalitic viruses invade the brain from the bloodstream. Tunicamycin could serve as a useful tool for use in such studies that could ultimately provide information which suggests ways by which blood-nervous system barriers may be protected to lessen the ability of viruses to enter the brain. Tunicamycin could also be useful as a means to alter the BBB in studies investigating the role of the BBB in mediating the process of neurodegeneration in a number of diseases.

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