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Astrocytes as targets for Venezuelan equine encephalitis virus infection

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Venezuelan equine encephalitis virus **(VEE) produces an acute infection in humans and induces a well-characterized cytopathic effect in neurons of the central nervous system (CNS). However, little is known about the role of glial cells in response to VEE infection of the CNS. Our results demonstrate that VEE is capable of a productive infection in primary astrocyte cultures and that this infection is cytotoxic. Further, there were significant differences in the growth kinetics comparing virulent and attenuated strains of VEE. Additionally, VEE infection of astrocyte cultures induced gene expression of two neuro-immune modulators, tumor necrosis factor-alpha (TNF-α) and inducible nitric oxide synthase (iNOS). Assays for TNF-α protein and nitric oxide (NO) demonstrated high levels of TNF-α protein and low levels of NO in response to VEE infection of astrocytes. These observations suggest an important role of astrocytes in this virus-induced encephalitis, and that interactions between astrocytes, other glial cells, and neurons may be important in VEE pathogenesis. Such interactions, which could impact neuronal survival, may include loss of functional changes in astrocytes or, alternatively, their production of neurotoxic molecules.**

Keywords: alphavirus; encephalitis; glia; cytokines

Introduction

Glial cells play a central role in neuronal function and viability. Astrocytes, the predominant glial cell type in the central nervous system (CNS), outnumber neurons by a ratio of 8:1 and carry out critical functions in normal CNS physiology including neuronal guidance during development (Juliano *et al*, 1996), structural processes for the blood-brain barrier (Janzer and Raff, 1987), buffering of potassium ions, removal of neurotransmitters, specifically glutamate (Keyser and Pellmar, 1994; Marrero and Orkand, 1996; Orkland and Opava, 1994; Vernadakis, 1996), and synthesis of essential neurotrophic factors (Gray and Patel, 1992; Muller *et al*, 1984; Schmalenbach and Muller, 1993; Wilkin *et al*, 1990; Yoshida and Gage, 1991). Dysfunction of the CNS can result from trauma, hypoxia, toxic chemicals, radiation, neurodegenerative diseases such as Parkinson's or Alzheimer's diseases, and certain neurotropic infections including virus infections. Regardless of the type of insult, neuronal degeneration appears to be due to a final common pathway (Dugan and Choi, 1994) that includes glial cell activation or dysfunction (Mucke and Eddleston, 1993). This results in loss of trophic support, production of free radicals, or excitotoxicity.

Venezuelan equine encephalitis virus (VEE) is a positive-sense RNA virus indigenous to Central, South and parts of North America. A member of the Togaviridae family, this mosquito-transmitted virus causes encephalitis in horses, but is also capable of causing disease in humans. The most recent outbreak of the disease occurred in 1995 where over 12 000 cases were reported in South America (MMWR, 1995). The characteristic clinical features of this infectious encephalitic disease include headache, fever, chills, skin rash and malaise. Clinical signs usually persist for 5–10 days, and encephalitis develops in approximately 0.4% of cases, however, children are particularly vulnerable as their occurrence of encephalitis is approximately

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4% (de la Monte *et al*, 1985; Kissling and Chamberlain, 1967).

The pathogenesis of VEE has been described as a primary infection of lymphocytes and neurons (Grieder *et al*, 1995, 1997; Grieder and Nguyen, 1996; Jackson *et al*, 1991; Jackson and Rossiter, 1997; Johnson *et al*, 1997). While it has been established that neurons in the CNS undergo degeneration following VEE infection (Charles *et al*, 1995; Grieder *et al*, 1995; Jackson *et al*, 1991; Jackson and Rossiter, 1997), the role of glial cells during the acute stage of VEE infection is unclear. Astrocytes are targets for other viral infections including *JC virus* (Aksamit *et al*, 1986), *Theiler's virus* (Aubert *et al*, 1987), *Visna virus* (Stowring *et al*, 1985), *Borna virus* (Carbone *et al*, 1991), as well as *Human Immunodeficiency virus* type-1 (Tornatore *et al*, 1994). However, these viruses induce slow, progressive diseases of the CNS, which differ from VEE in that VEE causes an acute, rapidly progressing disease. Understanding the responses of astrocytes in an acute infection and how these responses differ from slowly progressing infections is necessary for understanding acute inflammatory responses in the CNS.

Recently, an understanding of the unique immune system of the CNS has emerged. Previously, the CNS was described as 'immunologically privileged', due to the isolation of the CNS from immune surveillance by circulating lymphocytes because of the blood-brain barrier, and the absence of a lymphatic drainage system. Recognizing these differences in CNS immune system structure and function, a relatively new area of research has developed to characterize how the CNS responds to infections and injury. It is now understood that glial cells, specifically astrocytes and microglia, play a critical role in orchestrating the immune response of the CNS. Besides their physiologic role in CNS homeostasis, astrocytes can function as antigen presenting cells in association with major histocompatibility complex class I and class II molecules on their surface (Mucke and Eddleston, 1993; Neumann *et al*, 1996; Nikcevich *et al*, 1997), secrete cytokines and growth factors (Benveniste, 1992; Brodie *et al*, 1997; Chung and Benveniste, 1990; Clatterbuck *et al*, 1996; Lieberman *et al*, 1989; Mendez *et al*, 1997), phagocytose debris (Bechmann and Nitsch, 1997), and produce reactive oxygen and nitrogen intermediates (Banati *et al*, 1993; Brodie *et al*, 1997; Chao *et al*, 1996).

Our experiments characterize VEE infection of astrocytes *in vitro* and describe VEE replication kinetics and VEE induction of astrocyte cell death. Experiments were also conducted to determine if astrocytes could respond to VEE infection by the upregulation of certain pro-inflammatory molecules at the level of gene transcription and translation. Specifically, tumor necrosis factor-α (TNF-α) and inducible nitric oxide synthase (iNOS) were assessed by means of reverse transcriptase polymerase chain reaction (RT–PCR) and Southern blot analysis because of the documented production of these two molecules in response to various stimuli including interferon-gamma (IFNγ) or lipopolysaccharide (LPS) (Lowenstein *et al*, 1993; Xie *et al*, 1993) and certain neurotropic viruses (Lieberman *et al*, 1989). Furthermore, TNF-α protein secretion into the culture supernatant, as well as nitrite (NO^-_2), the stable oxidation product of nitric oxide (NO), were assessed by quantitative assays. These experiments form the basis for understanding the role of glial cells in early neuro-immune responses against this acute viral infection and the potential impact on neurons of the CNS.

Results

Astrocytes are targets for VEE infection

Astrocyte cultures infected with VEE for 18 h revealed a consistent pattern of morphological changes characteristic of apoptosis. Specifically, nuclei became concentric and fragmented, however no specific immunostaining for VEE could be visualized on the cell surface or in the cytoplasm (Figure 1B). At 24 h post-infection (p.i.), positive immunostaining for VEE antigen accompanied by early signs of cytolysis was evident (Figure 1C), and at 48 h positive immunostaining was most intense with severe cytopathic effects (CPE) of the monolayer (Figure 1D). In contrast, mock-infected control wells demonstrated healthy cell populations with defined centrally located nuclei and normal cell morphology throughout the experimental time course (Figure 1A).

Astrocytes support VEE replication

Primary astrocytes were infected with two molecularly cloned strains of VEE with different pathogenesis, a virulent strain (V3000) and an attenuated strain (V3010), to determine if astrocytes could support VEE replication and if the *in vivo* phenotype influenced replication in culture. Following VEE entry and removal of non-penetrated virus at 1 h p.i., both strains replicated rapidly in astrocytes with a peak of released virus at 24 h p.i. (Figure 2). However, the attenuated strain V3010 demonstrated slower replication rates as compared to the virulent V3000 and this difference was statistically different at 6 h p.i. ($^*P<0.05$). These data suggest that replication rates in astrocytes may contribute to the *in vivo* phenotype of VEE.

All extracellular VEE titers remained stable for the initial 48 h p.i. and then began to decline. This decline in VEE titers coincided with the microscopically observed CPE in VEE infected astrocytes (e.g., cytoplasmic granulation, cell shrinking and rounding) including the appearance of floating cellular debris in the culture supernatant. The slow

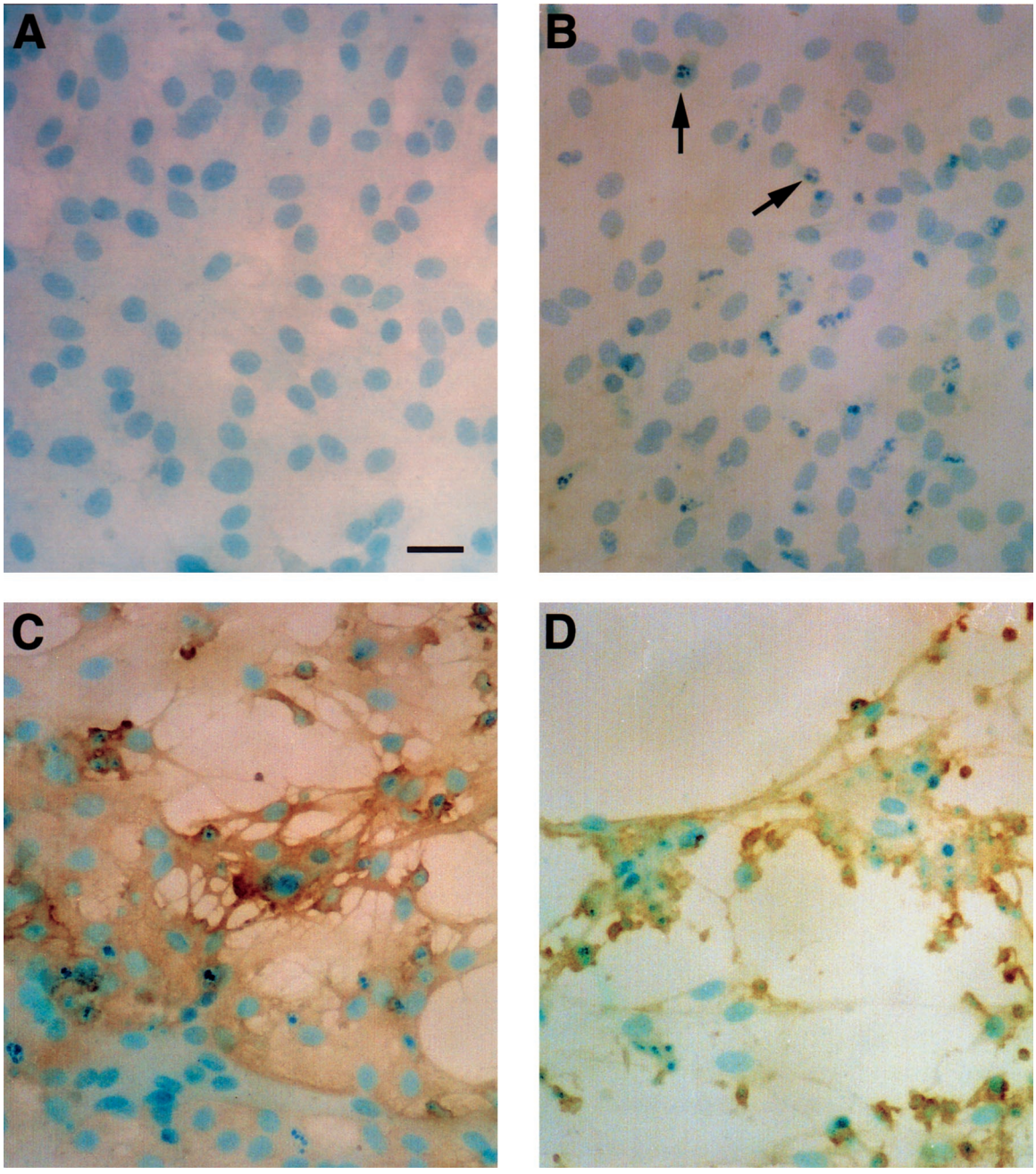

Figure 1 Photomicrograph of immunoperoxidase staining of astrocytes infected with VEE or mock-infected. Mock-infected controls show no staining (**A**). Early time points post-VEE infection (**B**, 18 h) demonstrate morphologic changes in astrocyte cultures including nuclear condensation and fragmentation (arrows), but no positive staining for VEE. Time points 24 and 48 h (**C** and **D**, respectively) reveal positive brown staining for VEE and cytopathic effects to primary astrocyte cultures. Scale bar=10 μm.

decrease in VEE titers in primary astrocyte culture supernatants (1.2 logs over 48 h) reflects the relative stability of this virus in non-cell-associated, fluid phase at 37°C.

Cytopathic effects of VEE infection

Although early morphologic changes were evident at 18 h p.i. (Figure 1B) there was not a decline in cell viability until 24 h p.i. (Figure 3). This appearance of CPE occurs at the same time as peak virus titers measured in the supernatant. While both VEE strains produced CPE in astrocytes, the attenuated strains resulted in delayed cytopathology. This trend of delayed CPE was not statistically significant. Despite similar peak virus titers and cytopathol-

ogy at 72 h p.i., differences in kinetics in replication and CPE in astrocytes may correlate with the *in vivo* phenotypes of the different VEE strains.

Induction of pro-inflammatory genes in astrocytes following VEE infection

Because of kinetic differences in replication rates for VEE and CPE in astrocytes we evaluated kinetics in gene induction for two pro-inflammatory molecules following infection with the molecularly cloned VEE strains V3000 and V3010. IFNγ/LPS treatment was used as a positive control. Both VEE strains (virulent V3000 and neuro-attenuated V3010) induced iNOS and TNF-α genes in astrocyte cultures in response to infection (Figure 4). Statistical analyses of triplicate samples indicated that IFNγ/LPS-stimulated astrocytes had statistically significant increases in iNOS gene expression as early as 6 h p.i. as compared to uninfected controls ($^*P<0.05$), whereas both virus-treated groups were not statistically significant from controls until 12 h p.i. (Figure 5). These levels of iNOS gene induction peaked at 24 h p.i. and remained higher throughout the remainder of the experiment for all three groups as compared to uninfected controls.

Comparisons among groups indicated that the IFNγ/LPS-stimulated astrocytes upregulated gene expression for TNF-α as early as 2 h post-stimulation and then declined, whereas the virus-infected astrocytes showed slower upregulation of TNF-α

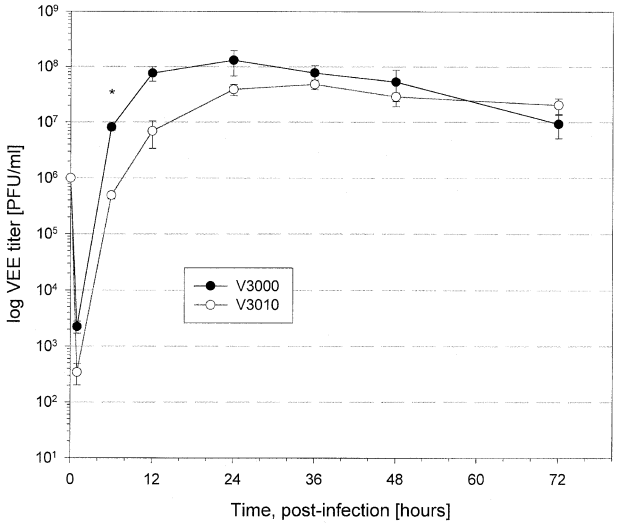

Figure 2 Astrocyte cultures were infected at an MOI of 1.0, and VEE titers in the culture supernatant were determined at 1, 6, 12, 24, 36, 48 and 72 h p.i. Virulent and attenuated VEE strains demonstrate rapid, logarithmic growth within the first 24 h. Attenuated V3010 demonstrated a statistically significant slower rate of replication at 6 h p.i. ($^*P<0.05$) as compared to virulent V3000.

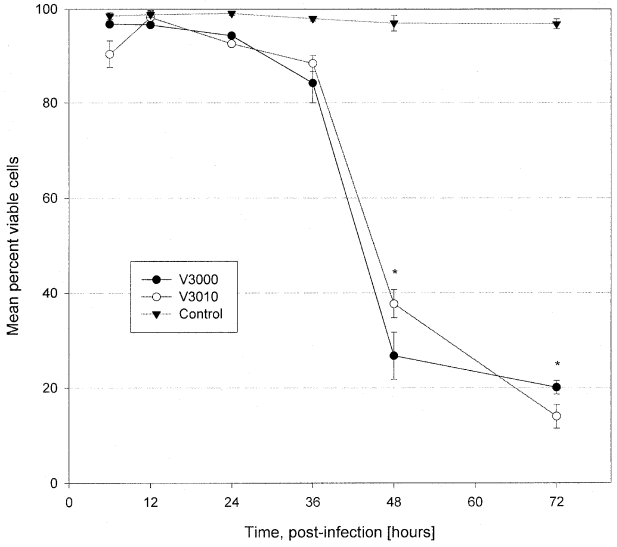

Figure 3 Astrocyte cultures were infected at an MOI of 1.0 with virulent and attenuated VEE strains and cell viability was determined by Trypan Blue exclusion test. Mean per cent $\pm$s.e.m. of viable cells was determined by three independent samplings along with mock controls for each time point. Both VEE strains were cytopathic for astrocytes ($^*P<0.05$) as compared to uninfected controls.

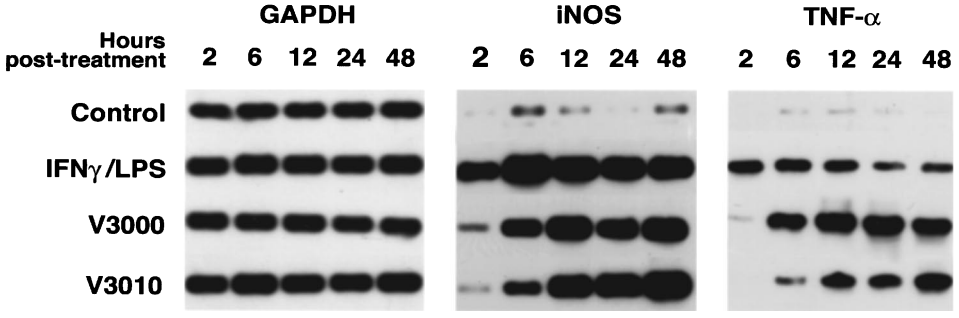

Figure 4 Southern blots for gene expression of GAPDH, iNOS, and TNF-α. Astrocytes were infected with virulent V3000 or neuroinvasive, attenuated clone V3010 at an MOI of 1.0. Both strains of VEE demonstrate gene induction as early as 6 h p.i. The housekeeping gene GAPDH remains unchanged throughout the experiment.

message RNA (Figure 6). This upregulation for TNF-α gene expression was statistically significant for V3000 as early as 6 h p.i. as compared to uninfected controls ($*P<0.05$). These levels of TNF-α gene induction remained higher throughout the experiment for both virus-infected groups as compared to uninfected controls. Similar to results previously described by others (Chung and Benveniste, 1990), our experiments demonstrated that a combination of IFNγ and LPS had a synergistic effect on gene expression in astrocytes when compared to individual stimulatory effects by IFNγ or LPS (data not shown). Finally, our data demonstrate that astrocyte infection with VEE induced consistent levels of TNF-α gene expression when compared to IFNγ/LPS and that levels of TNF-α gene expression were highest for the virulent V3000.

Production of NO and TNF-α in astroyctes in response to VEE infection

Nitrite assays of culture supernatants from primary astrocytes infected with VEE or stimulated with IFNγ/LPS demonstrated that nitric oxide was produced at statistically significant levels ($*P<0.05$, Figure 7) as compared to uninfected controls. Nitrite levels in identical astrocyte cultures infected with either the virulent or the neuroattenuated VEE clone remained at base line for the first 24 h p.i. and were only elevated at low levels (5–6 μM/ml) at 48 h p.i. These low levels of nitrite in VEE-infected astrocytes were statistically significant as compared to uninfected controls ($*P<0.05$). Interestingly, there was no difference in NO production between the virulent and attenuated VEE-infected astrocytes. In contrast, the NO production in the positive control treated astrocyte cultures (IFNγ/LPS) resulted in elevated nitrite levels as early as 12 h post-stimulation ($*P<0.05$) and nitrite levels continuously increased over the experimental time course.

Immunoassays of these culture supernatants demonstrated that these cells secreted significant amounts of TNF-α when compared to untreated controls ($*P<0.05$, Figure 8). Infection of identical astrocyte cultures with virulent V3000 resulted in

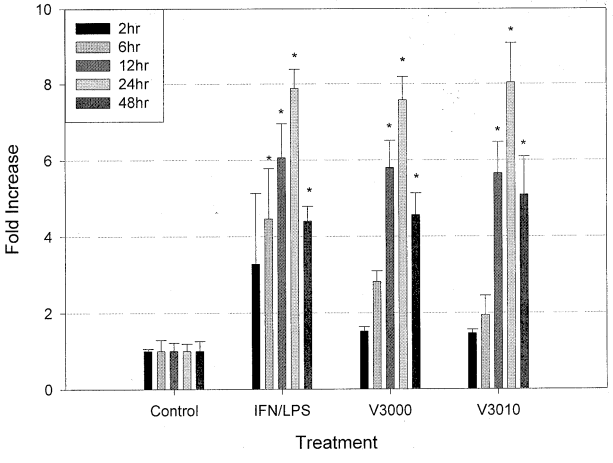

Figure 5 Histogram depicting changes in gene expression for iNOS based on optical density measurements in response to VEE infection. Bars represent fold increase as compared to untreated controls (means ± s.e.m.) of three independent samples. Both virulent and attenuated strains for VEE, as well as IFNγ/LPS, were capable of significant induction for iNOS ($*P<0.05$).

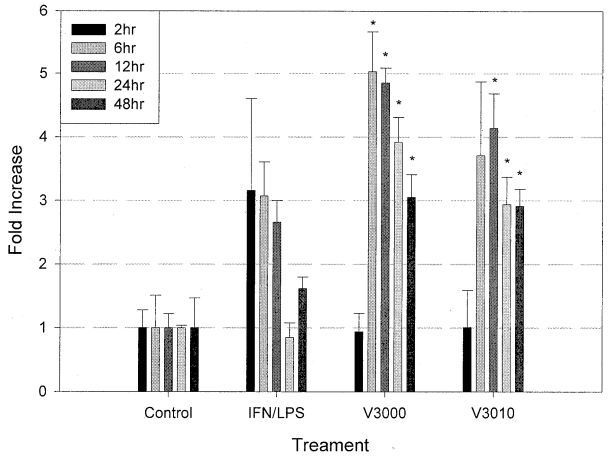

Figure 6 Histogram (mean ± s.e.m. of three independent samples) depicting changes in gene expression for TNF-α based in optical density measurements in response to VEE infection. Virulent and attenuated VEE significantly induced TNF-α gene expression in primary astrocytes ($*P<0.05$) as compared to uninfected controls. Virulent V3000 upregulated TNF-α gene expression as early as 6 h p.i., and levels of TNF-α gene expression in both virus genotypes were higher as compared to IFNγ/LPS stimulated astrocytes.

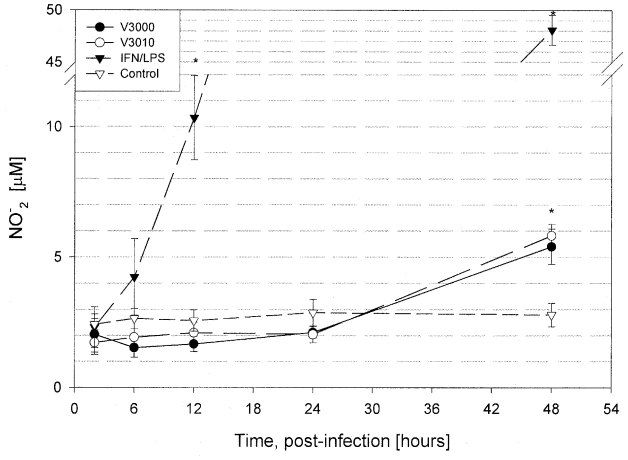

Figure 7 Astrocytes infected with virulent V3000 or neuroattenuated V3010 or stimulated with IFNγ/LPS showed very different kinetics and levels of nitric oxide production. IFNγ/LPS stimulated astrocytes produced high levels of nitrite that resulted in final concentrations of 48 μM nitrite at 48 h, whereas VEE-infected astrocytes produced low levels of nitrite (5 μM) that were only detectable at 48 h p.i. ($*P<0.05$ when compared to uninfected controls).

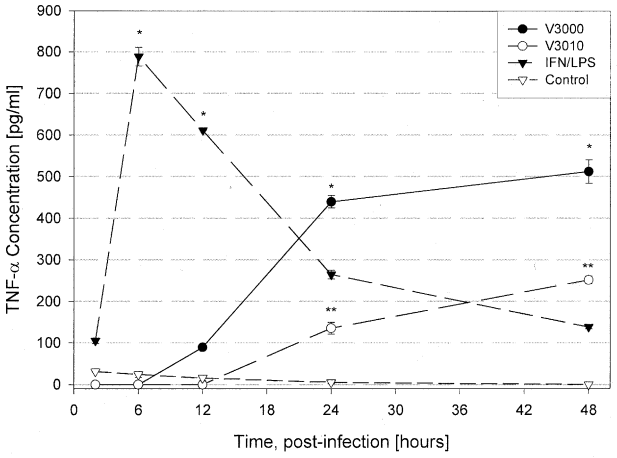

Figure 8 Astrocyte supernatants were analzyed for TNF-α protein levels following infection with virulent V3000 or neuro-attenuated V3010 or stimulated with IFNγ/LPS. IFNγ/LPS-stimulated astrocyte production of TNF-α peaked at 6 h post-stimulation (*$P<0.05$) and then declined to near baseline levels by 48 h. VEE-infected astrocyte production of TNF-α peaked at 48 h p.i. (*$P<0.05$). Neuro-attenuated V3010 infection of astrocytes resulted in significantly lower levels of TNF-α as compared to virulent V3000 at 24 and 48 h p.i. (**$P<0.05$).

secreted TNF-α levels of 500 pg/ml, but this peak was not reached until 48 h p.i. Infection with the neuro-attenuated V3010 resulted in similar TNF-α secretion kinetics, however the levels of TNF-α measured in the supernatants were significantly reduced as compared to V3000 (**$P<0.05$). Primary astrocyte cultures stimulated with IFNγ/LPS were used as positive controls and secreted 800 pg/ml of TNF-α as early as 6 h post-stimulation (*$P<0.05$). This peak sharply declined over the next 24 h and returned to near baseline levels by 48 h post-stimulation.

Discussion

VEE is capable of infecting primary astrocytes and replicating efficiently, with the consequences of causing substantial cell death. It is well documented that VEE is neuro-virulent (Charles *et al*, 1995; Grieder *et al*, 1995; Jackson *et al*, 1991; Jackson and Rossiter, 1997), however, our *in vitro* experiments, as well as *in vivo* experiments from Jackson and Rossiter (1997), suggest that astrocytes are a potential target for VEE after it has established a productive infection in the CNS. If substantial numbers of astrocytes undergo cell death following VEE infection *in vivo*, these astrocyte-VEE interactions could be a contributing factor to neurodegeneration because essential astrocyte functions are lost. Alternatively, if during the course of VEE infection astrocytes are activated to produce pro-inflammatory cytokines and other diffusible molecular immuno-modulators, this response may be detrimental to neighboring neurons.

Pro-inflammatory responses have been associated with acute neurodegeneration in several different models, including traumatic brain injury and ischemia (Rothwell and Strijbos, 1995) and systemic bacterial infections (Waage *et al*, 1989). Further, inflammatory responses in the CNS have also been linked to specific types of neurological disease, such as autoimmune processes in multiple sclerosis (Benveniste, 1992), AIDS dementia (Adamson *et al*, 1996; Talley *et al*, 1995; Yoshioka *et al*, 1995), or other CNS viral infections (Lieberman *et al*, 1989). Astrocytes and other glial cells during the inflammatory response in the CNS may influence the balance between host protection and neurotoxicity.

Neuro-immune responses of astrocytes to infection by two phenotypically different VEE strains demonstrate that the expression of two pro-inflammatory genes, iNOS and TNF-α, are induced when compared to uninfected control cultures. These two VEE strains, the molecularly cloned virulent V3000 and the neuro-invasive, but attenuated clone, V3010, differ in their genotype by one single nucleotide (Davis *et al*, 1991). These two molecularly cloned VEE strains with only a single amino acid difference that results in extremely different *in vivo* phenotypes allow us to elucidate cellular responses to infection including cytotoxicity and gene induction in a variety of potential target cell populations. Our finding of slower replication rates in astrocytes for V3010 supports previous findings that slower viral replication rates of V3010 in the periphery may play a role in the attenuated phenotype (Grieder *et al*, 1995).

In previous work, investigating pro-inflammatory gene induction in the periphery in response to VEE infection, we demonstrated that two molecularly cloned VEE strains elicited different cytokine responses when compared to one another. Specifically, an attenuated strain resulted in slower induction kinetics, suggesting that a delayed cytokine response may influence the development of host-protection (Grieder *et al*, 1997). In contrast, the present results indicated that two neuro-invasive VEE clones are capable of strongly inducing two important genes in astrocytes, and that the kinetics of gene expression are similar. These similarities in iNOS and TNF-α gene expression following virulent and attenuated VEE infection *in vitro*, yet very different mortality rates following infection with different VEE phenotypes *in vivo* suggest that other factors are involved in the CNS pathogenesis. In the present study, immunoassays for TNF-α demonstrate that additional regulatory mechanisms, such as post-transcriptional events or protein function regulation are important in the final outcome of TNF-α secretion into the supernatant. Further support for

post-transcriptional regulation is the observation of low nitrite levels in VEE-infected astrocyte cultures. Levels of nitrite were much lower than predicted given the quantitation of iNOS gene expression in virus-infected and IFNγ/LPS-induced control astrocytes. Possible explanations for post-transcriptional regulation of iNOS include the availability of substrates and cofactors essential for the synthesis of iNOS and NO, or other unknown regulatory factors. Such post-transcriptional regulation of iNOS has been demonstrated in other immunocompetent cells (Le Page *et al*, 1996). These observations support the concept that the final outcome of CNS infection with VEE of different phenotypes may not only depend upon differences in virus replication rates and dissemination, but also upon other cell targets such as astrocytes or microglia, and the induction of pro-inflammatory genes at the level of expression or post-transcriptional regulation.

Other investigators have determined pro-inflammatory responses in the brain following infections with closely related viruses. Griffin and colleagues in 1994 characterized cytokine expression in the brain in response to the alphavirus *Sindbis virus* (SB) (Tucker *et al*, 1996; Wesselingh *et al*, 1994). Their results demonstrated that pro-inflammatory cytokines play a significant role in the pathogenesis of SB-induced encephalitis and, specifically, that NO may be involved in protecting mice from fatal progression of the disease. *Sindbis virus* has also been utilized to characterize cytokine responses in murine astrocytes *in vitro* (Brodie *et al*, 1997). In contrast to the SB *in vivo* studies, these investigators found that neuro-virulent SB infection in astrocytes induced TNF-α expression, but not iNOS, and the authors concluded that SB infection of astrocytes does not produce enough TNF-α to induce iNOS gene expression. Differences between our findings with VEE and SB infection of astrocytes may include host species differences, differences between molecularly cloned VEE and biological mutants of SB, and difference in the sensitivity of quantitative methods (i.e. semi-quantitative PCR for VEE *versus* Western blots for SB).

Studies investigating *Japanese encephalitis virus* (JEV), a closely related virus in the family Flaviviridae, supports the beneficial role of NO in viral encephalitis (Lin *et al*, 1997) both *in vivo* and *in vitro*. *In vitro*, inhibition of replication of JEV in IFNγ-activated murine macrophages was correlated to cellular NO production. *In vivo*, the mortality rate increased as the JEV-infected mice were administered a NOS competitive inhibitor. However, these results with JEV are in contrast to results describing experiments involving another Flavivirus, *Tick-borne encephalitis virus* (TBE) (Kreil and Eibl, 1996). Macrophages from TBE-infected mice spontaneously produced NO *in vitro* and high levels of NO production did not display an inhibitory influence on TBE replication. *In vivo* administration of a competitive inhibitor of NO production to TBE-infected mice significantly increased their mean survival time. These data suggest that NO plays a role in the development of TBE disease and that inhibition of NO formation may be beneficial to the host. These contrasting findings suggest that inducible genes, specifically iNOS, in the CNS have unique responses to similar types of challenges and that there is a low threshold from host protection to toxicity. Further analysis with sensitive quantitative and qualitative methods is necessary to unravel the role of iNOS and NO production, as well as other pro-inflammatory mediators, following viral infections of the CNS.

Primary astrocyte cultures are an important tool in understanding the relationship between glial cells, neurotropic viruses, and cytokines or other diffusable molecules. These highly homogenous cultures are useful in isolating responses that may occur during the neurotropic phase of VEE infection *in vivo*. The benefits of using astrocyte cultures as an experimental model for studying CNS viral infections include characterizing replication rates, determining cellular outcomes and neuro-immune responses. Similar models have been used for other neurotropic viruses including *Japanese encephalitis virus* (Lin *et al*, 1997; Suri and Banerjee, 1995), *Sindbis virus* (Brodie *et al*, 1997), *Tick-borne encephalitis virus* (Kreil and Eibl, 1996), and the paramyxovirus *Newcastle disease virus* (Fisher *et al*, 1994; Lieberman *et al*, 1989, 1990; Rus *et al*, 1992). Moreover, astrocyte cultures have been used to characterize astrocyte responses to specific stimuli aimed at the upregulation of certain molecular factors including NO and TNF-α. Such inducers of NO and TNF-α in astrocytes include IFNγ, LPS, as well as neurotropic viruses such as *Newcastle disease virus* and *Theiler virus* (Chung and Benveniste, 1990; Feinstein *et al*, 1996; Galea *et al*, 1994; Molina-Holgado *et al*, 1997; Rus *et al*, 1992). Combining primary glial cultures and molecularly cloned VEE with well-characterized phenotypes will help further this progress.

In summary, our results demonstrate that VEE infects astrocytes *in vitro* and that the response of these primary astrocytes to VEE infection includes cytotoxic effects and the induction of pro-inflammatory genes. Because of these findings *in vitro* and the fact that VEE is neuro-invasive, astrocytes are likely targets for infection after VEE has crossed the blood-brain barrier. Even if only a small subset of astrocytes are infected, because of the efficient replication rates, infected astrocytes may play a significant role in terms of increasing intracerebral viral titers and inducing specific neuro-immune mediators. Astrocyte infection and activation could therefore contribute to the pathogenesis of VEE encephalitis. Understanding the relationship of

early neuro-inflammatory responses to VEE infection may help in targeting and manipulation of such responses that are beneficial. Such strategies could have significant application for a variety of neurodegenerative processes that share similar pathways, responses and outcomes.

Materials and methods

Virus
Two molecularly cloned VEE, virulent V3000 and attenuated V3010, were used (Davis *et al*, 1991; Grieder *et al*, 1995). The virulent V3000 and neuroinvasive, attenuated clone, V3010 differ in their genotype by one single nucleotide resulting in a single amino acid change at glycoprotein E2 position 76. This nucleotide mutation replaces a glutamic acid in V3000 for lysine in V3010. Virulent V3000 is 100% fatal in mice regardless of route of injection. Attenuated V3010 is nonlethal when injected peripherally, and only has a 20% mortality rate when injected intracerebrally (Grieder *et al*, 1995). Molecularly cloned virus stocks were stored at -80°C and all experiments were conducted in a biosafety level 3 laboratory.

Primary astrocyte cultures
Primary astrocyte cultures were established as previously described (McCarthy and de Vellis, 1980). Briefly, cerebrums were removed from 2-day-old Sprague-Dawley rats using sterile technique. Meninges were stripped by microscopic dissection and cerebral hemispheres were dissociated by suction pipetting followed by centrifugation at 1000 r.p.m for 10 min. The supernatant was removed and the cell pellet was resuspended in cell culture media. This suspension was then triturated sequentially through 18 gauge and 22 gauge needles, and cells were plated in Dulbecco's minimum essential media (DMEM) supplemented with 10% non-heat inactivated fetal bovine serum (FBS), 1% L-glutamine, and 25 μg/ml gentamycin. After the establishment of a confluent monolayer (between 10–14 days), adherent microglia were removed by rotary shaker. The resulting cultures were characterized as astrocytes by positive immunofluorescent staining for glial fibrillary acidic protein (GFAP; Sigma, St. Louis, MO, USA), an intermediate filament which is expressed by astrocytes. The homogeneity of these astrocytes was determined by immunostaining identical cultures with a microglia specific cell-surface marker antibody OX42 (Serotec/Harlan; Indianapolis, IN, USA) and an oligodendrocyte specific antibody antiGal-C (Boehringer, Germany). The astrocyte primary cell cultures were determined to be greater than 95% homogenous by three independent samplings of GFAP stained cell cultures, and comparing those cell counts to phase contrast light microscopy. Contaminating microglia and oligodendrocytes were observed only on rare occasions.

Immunoperoxidase staining of VEE-infected astrocytes
Astrocyte cultures were passaged one time and plated into 16-well tissue culture chambers (Nunc Inc., Naperville, IL, USA) and incubated for 48 h at 37°C, 5% CO_2. Cell counts at this time were determined to be 1.5×10^5 cells per well. Monolayers were infected with VEE at a multiplicity of infection (MOI) of 1.0. Mock controls were inoculated with phosphate buffered saline (PBS) only. Following 1, 6, 12, 18, 24, or 48 h infection the cells were fixed in an ice cold 1 : 1 mixture of methanol and acetone for 5 min and allowed to air dry. Slides were treated with a 1 : 400 dilution of polyclonal rabbit anti-VEE serum (kindly provided by Drs George Ludwig and Jonathan Smith, USAMRIID, Ft. Detrick, MD, USA) and stained using an avidin-biotin-conjugated peroxidase staining kit (Vectastain ABC Kit, Burlingame, CA, USA) with diaminobenzidine (DAB) used as the chromagen resulting in brown staining (Grieder and Nguyen, 1996). Cells were double-stained with methylene blue to visualize nuclear structures. Controls for non-specific staining included stained mock-infected cells and infected cells treated with unimmunized rabbit serum incubated at the same dilution as the anti-VEE rabbit serum.

Virus growth kinetics
Astrocytes were passaged one time and plated into 60 mm cell culture plates (Corning, NY, USA). Cultures were incubated for 5 days at 37°C, 5% CO_2 until 90% confluent. Cell counts at this time were determined to be 1.5×10^6 per 60-mm cell culture plate. Triplicate astrocyte cell cultures were then infected with virulent V3000 or the attenuated V3010 virus at an MOI of 1.0 and incubated for 1 h at 37°C, 5% CO_2. Astrocytes were washed three times with PBS containing 0.1% donor calf serum (DCS) and 3 ml of media was replaced. The initial samples were collected at this time, and subsequently at 6, 12, 24, 36, 48, and 72 h p.i. Supernatant samples containing virus were immediately frozen at -80°C. Virus titers were determined as previously described by plaque assays on BHK-21 cells (American Type Culture Collection, Rockville, MD, USA) (Grieder and Nguyen, 1996; Scherer *et al*, 1971). Virus titers were calculated as plaque forming units (PFUs) per ml of supernatant.

Astrocyte viability
Astrocytes were passaged one time, plated into 6-well culture plates (Corning, NY, USA) and incubated at 37°C, 5% CO_2 for 4 days. Cell counts were determined to be 3×10^5 cells per well. Medium was removed and three independent wells

of astrocytes were infected with V3000 or V3010 virus at an MOI of 1.0 and incubated at 37°C, 5% CO_2 for 1 h. Mock-infected controls were treated with PBS only for 1 h. Infected and control astrocytes were harvested at 6, 12, 24, 36, 48, and 72 h p.i. Briefly, the astrocyte medium was removed and the monolayers were trypsinized. Trypsin action was neutralized with warm astrocyte medium containing 10% FBS and triturated to lift the monolayer. Cells were stained with Trypan Blue (Life Technologies, Inc., Grand Island, NY, USA), counted by hemocytometer, and mean percentage ($\pm$s.e.m.) of living cells calculated.

Extraction of astrocyte mRNA

Astrocytes were passaged one time and plated into 6-well cultures plates and incubated at 37°C, 5% CO_2 for 2 days. Cell counts were determined to be 1×10^6 cells per well. Medium was removed and wells were assigned to one of the following groups: (1) uninfected PBS control; (2) a combination of recombinant rat IFNγ (100 U/ml; Genzyme Corp, Cambridge, MA, USA) and LPS stimulated (1 μg/ml equals 200 U/ml; protein free *E. coli* K235 LPS extracted by the method of McIntire *et al* (1967) kindly provided by Dr Stefanie Vogel, USUHS, Bethesda, MD, USA); and (3) infected with V3000 or V3010 at an MOI of 1.0 and incubated for 1 h. Total cellular RNA was harvested from three independent samples per treatment group at 2, 6, 12, 24, and 48 h p.i. using RNAzol™ B (Tel-Test, Inc., Friendswood, TX, USA) as previously described (Chomczynski and Sacchi, 1987). RNA was extracted with chloroform (Sigma, St. Louis, MO, USA), precipitated with isopropanol (Sigma, St. Louis, MO, USA) and diluted in diethylepyrocarbonate (DEPC)-treated water (Quality Biologicals, Inc., Gaithersburg, MD, USA). The concentration of RNA in each sample was determined using a spectrophotometer (Beckman Instruments, Inc., Columbia, MD, USA). Specimens were stored at -80°C until processing by RT–PCR.

RT–PCR detection of mRNA

cDNA synthesis was performed by reverse transcription in a reaction volume of 25 μl using: (1) 1 μg of RNA; (2) random hexamer oligonucleotides at a concentration of 0.5 U; (3) 0.25 mM mix of all four deoxynucleotide triphosphates (dNTPs); (4) 1 $\times$ reverse transcriptase buffer (50 mM Tris-HCL, pH 8.3, 75 mM KCL, 3 mM $MgCl_2$); 8 mM DTT; and 200 U Moloney Murine Leukemia virus (MMLV) reverse transcriptase (GIBCO, Gaithersburg, MD, USA). The reaction mixture was incubated at 37°C for 60 min, heated to 90°C for 5 min to denature the enzyme and cooled on ice before storage at -20°C. The final reaction volume was diluted with an addition 175 μl of DEPC treated water.

Amplification of cDNA was accomplished using gene-specific sense and antisense oligonucleotide primers for rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH), TNF-α, and iNOS (Table 1). Products amplified from cDNA could be distinguished from genomic DNA because the primers were designed to span at least one intron. To each PCR reaction the following components were added: (1) 1.0 mM dNTP mix; (2) 1 $\times$ PCR buffer (50 mM KCL, 10 mM Tris-HCL, 3 mM $MgCl_2$); (3) 10 μl of cDNA; (4) 0.2 μM sense and antisense primers; (5) and 1 U Taq polymerase (Promega, Madison, WI, USA). PCR reaction mixture was then amplified using an automated PCR thermocycler (Perkin-Elmer, Norwalk, CT, USA). Ten μl of amplified PCR products along with 2 μl of gel

Table 1 Oligonucleotides specific for rat used in PCR and Southern blots

Product	*Primer/probe*	*Sequence (5′ to 3′ direction)*	*Product size*
GAPDH	sense	CCATGGAGAAGGCTGGGG	195 bp
	antisense	CAAAGTTGTCATGGATGACC	
	probe	CTAAGCATGTGGTGGTGCA[1]	
TNF-α	sense	AGAACTCCAGGCGGTGTCTGT	365 bp
	antisense	CCTTGTCCCTTGAAGAGAACC	
	probe	ATCAGTTCCATGGCCCAGA	
iNOS	sense	CTGCATGGAACAGTATAAGGCAAAC	229 bp
	antisense	GAGACAGTTTCTGGTCGATGTCATGA	
	probe	GGGCTCCAGCATGTACC	

PCR amplification specifications				
Product	*Cycle number*	*Annealing temperature*	*Accession number*	*Reference*
GAPDH	30	55°C	M17701	(Tso *et al*, 1985)
TNF-α	28	60°C	L19123	(Kirisits *et al*, 1994; Shirai *et al*, 1989)
iNOS	30	63°C	U03699	(Feinstein *et al*, 1996; Galea *et al*, 1994)

[1]This was the sequence of the probe used to detect GAPDH sequences following RT–PCR, however it should be noted that this sequence includes two mismatched nucleotides as compared to the original RNA sequence. The correct sequence should read CTAAGCAGTTGGTGGTGCA.

loading buffer were added to each well in a 1.5% agarose gel and electrophoresed at 90 V for 60 min in 1 × Tris buffer. After electrophoresis, the gels were denatured, neutralized and transferred to Hybond N^+ membranes (Amersham Life Science, Arlington Heights, IL, USA) using 10 × SSC by standard capillary Southern blotting techniques (Southern, 1975). DNA was then cross-linked to the membrane by exposure to UV light for 2 min and baked at 80°C in a vacuum oven. Subsequent visualization of specific DNA bands on the blots was conducted using fluorescein labeled-oligonucleotide probes complimentary to the PCR products and detected using the enhanced chemical luminescence technique (ECL™) (Amersham LifeScience, Buckinghamshire, England). Light output was detected on Hyperfilm™ ECL™ film (Amersham LifeScience, Buckinghamshire, England), that was then scanned into a digital image. Blots were quantified by obtaining pixel densities using Scion Image software for Windows (Scion Corporation, Frederick, MD, USA) and normalizing relative changes in gene expression of each treatment group at each time point to their matched untreated controls. GAPDH signals were consistent for all experimental treatment groups. It is important to note that this procedure only allows for relative quantitation and comparisons of relationships among treatment groups and does not provide direct comparisons with exact levels (i.e., units) of mRNA among treatment groups (Wynn *et al*, 1993).

TNF-α immunoassay

TNF-α protein in primary astrocyte cultures supernatant was measured using the Quantikine® M rat TNF-α kit (R&D Systems, Minneapolis, MN, USA) as per manufacturer's instructions. Briefly, astrocyte cultures plated at 10^6 cells/well were infected with one of the molecularly cloned VEE strains at an MOI of 1.0 or treated with IFNγ/LPS. Untreated astrocytes were used as a negative control. Supernatants were harvested from three independent wells for each treatment group at 2, 6, 12, 24, and 48 h p.i., and media replaced after each sampling. All samples were stored at 4°C and diluted 1 : 2 with the calibrator diluent before assaying to bring TNF-α levels within the range of the standards. Standards, controls, and samples were assayed in duplicate at a wavelength of 450 nm and 550 nm for wavelength correction. Optical density (O.D.) was determined as the change in O.D. between the two wavelengths and intensity of the color reaction product was quantitated on a microplate reader (ELx800, Biotek Instruments, Inc., Winooski, VT, USA) using Kineticalc software for Windows® (Version 1.5, Biotek Instruments, Inc., Winooski, VT, USA). A range of TNF-α dilutions was used to generate a standard curve to determine TNF-α concentrations in the sample supernatant.

Nitrite assay procedure

The amount of nitric oxide produced by astrocytes was determined by assaying its stable oxidation product, NO^-_2 (nitrite) (Green *et al*, 1982). Briefly, equal volumes (100 μl) of sample and Griess reagent (1% sulfanilamide, 0.1% *N*-1-naphthyethylenediamine dihydrochloride in 2.5% H_3PO_4) (Sigma, St. Louis, MO, USA) were mixed in a 96-well plate. The optical density of the color reaction product from three independent samples was measured at 540 nm with a microplater reader (ELx800, Biotek Instruments, Winooski, VT, USA). A range of sodium nitrite dilutions was used to generate a standard curve. Values were quantified using Kineticalc software for Windows® (Version 1.5, Biotek Instruments, Inc., Winooski, VT, USA).

Statistical analysis

Data were analyzed using the software program SPSS for Windows©, version 8.0. VEE growth curves (Figure 2) data were analyzed using separate analysis of variances (ANOVAs) at 6, 12, and 24 h p.i. to determine whether there were treatment group effects. Least Significant Difference (LSD) *post hoc* tests were used to compare differences among groups at these time points. Cell viability data (Figure 3) were analyzed using repeated-measures ANOVA with the within-subjects factor of time and between-subjects factor of treatment in order to determine whether treatment groups differed over time. Separate ANOVAs were then conducted at 24, 48, and 72 h to determine whether groups differed significantly at these time points. LSD *post hoc* tests were used to compare differences among groups at these time points. Gene expression data (Figures 5 and 6) were normalized based on controls and analyzed using ANOVAs at each time point with the factor of treatment in order to determine whether groups differed based on treatment. Dunnett's *post hoc* tests were used to determine which treatment groups differed significantly from controls. These data also were analyzed with ANOVAs performed on each treatment group with the factor of time in order to determine whether treatment effects differed depending on time point measured. Dunnett's *post hoc* tests were also used to determine which time points within a specific treatment group (i.e., groups measured at 6, 12, 24, and 48 h) differed from the group measured at 2 h. Assays to determine NO and TNF-α production (Figures 7 and 8) were analyzed using separate ANOVAs at 6, 12, 24, and 48 h in order to determine treatment effect. LSD *post hoc* analyses were then used to determine statistical differences among treatment groups. ANOVAs were two-tailed with $P<0.05$, whereas *post hoc* tests were one-tailed because directionality (e.g., increase in gene expression) had been predicted with $P<0.05$.

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