The Immune Response to Herpes Simplex Virus Encephalitis in Mice Is Modulated by Dietary Vitamin E

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Abstract
Herpes simplex virus encephalitis (HSE) is the most common fatal sporadic encephalitis in humans. HSE is primarily caused by herpes simplex virus (HSV)-1 infection of the brain. HSE results in increased levels of oxidative stress, including the production of reactive oxygen species, free radicals, and neuroinflammation. The most biologically active form of vitamin E (VE) is α-tocopherol (α-TOC). In cellular membranes, α-TOC prevents lipid peroxidation by scavenging free radicals and functioning as an antioxidant. Supplementation with VE has been shown to decrease immunosenescence, improve immune function, and may be neuroprotective. To determine how VE deficiency and VE supplementation would alter the pathogenesis of HSE, we placed weanling male BALB/cByJ mice on VE-deficient (VE-D), VE-adequate (VE-A), or VE-supplemented diets for 4 wk, and then infected the mice intranasally with HSV-1. VE-D mice had more severe symptoms of encephalitis than VE-A mice, including weight loss, keratitis, hunched posture, and morbidity. VE-D mice had increased cytokine and chemokine expression in the brain and increased viral titers. In contrast, VE supplementation failed to decrease cytokine production and had no effect on viral titer. We demonstrated that adequate levels of VE are important in limiting HSE pathology and that 10× supplementation does not enhance protection.

Introduction
Herpes simplex virus encephalitis (HSE) is the most common fatal sporadic encephalitis in humans (1–3). Ninety percent of all HSE cases are caused by herpes simplex virus (HSV)-1 (4). Untreated, HSE has a 70% mortality rate. Treatment with antiviral medication, such as acyclovir, decreases HSE-associated mortality to 20%; however, only 38% of HSE patients recover to normal function (4,5). HSE is a substantial problem for the immunosuppressed, including people with HIV and those undergoing chemotherapy.

When administered intranasally (i.n.), HSV-1 enters the central nervous system (CNS) along neuronal pathways of the olfactory and trigeminal nerves (6). This route of infection results in an acute necrotizing encephalitis involving the olfactory and limbic systems, including the olfactory bulb, hypothalamus, thalamus, amygdala, hippocampus, and olfactory and entorhinal cortices. In this model, HSV-1 primarily infects neurons and glial cells (7,8). The infection of neurons and glia induce the production of proinflammatory cytokines produced by microglia and infiltrating macrophages, as well as the production of chemokines and antiviral cytokines (9,10). As virus replication continues, both CD4+ and CD8+ T lymphocytes infiltrate the brain (11–13). The intranasal route of infection mimics the hypothesized route of human HSE, where it is believed that the virus enters the CNS via the olfactory pathway or via the trigeminal ganglion (1,14,15). The intranasal model of HSV-1 infection has been well characterized in mice (8,13,16–19).

Vitamin E (VE) is a family of tocopherols and tocotrienols, of which α-tocopherol (α-TOC) is the most biologically active and second most abundant in food (20). These lipid soluble antioxidants vitamins are found in cellular membranes and prevent lipid peroxidation by scavenging free radicals (21). Deficiency in VE is associated with increased oxidative stress, central and peripheral neuropathies, and impaired immune function (22–24). VE deficiency increases the parasite load and pathology in mice that are experimentally infected with *Heligmosomoides polygyrus* (23). VE deficiency also decreases T- and B-cell numbers in rats infected with *Trypanosoma cruzi* (24).

Supplementation with VE has been shown to decrease immunosenescence, improve immune function, and may be neuroprotective. VE supplementation is capable of modulating T-cell cytokines, including interferon (IFN)-γ (25,26). Short-term, high-dose VE supplementation in colorectal cancer patients increases the production of both IFNγ and interleukin (IL)-2 (25). High dietary VE increases IFNγ and IL-2 production in aged mice after an influenza infection (26). In a recent study,
Han et al. (27) determined that VE affects a wide range of immune-related genes in old, but not young, mice. Additionally, VE has been shown to stop the age-related decline in the formation of CD4 T-cell synapses (28). In young restraint-stressed mice, VE has been shown to increase the production of IFN-γ and IL-2 in concanavalin A–stimulated splenocytes (29).

Studies from our laboratory have pointed to a key role for antioxidant micronutrients, including VE, in the pathogenesis of infectious diseases (30–34). Specifically, we showed that VE supplementation is capable of decreasing coxsackievirus-induced myocarditis in selenium-deficient mice. In the absence of VE, iron-loaded mice significantly increased coxsackievirus-induced myocarditis compared with iron-loaded, VE-adequate (VE-A) mice. Together, studies from our laboratory and others indicate that VE has the potential to modulate the immune response to a viral pathogen.

Because the brain is rich in lipids, we hypothesized that a deficiency in VE would increase HSE pathology in mice, and furthermore, VE supplementation would reduce the symptoms of HSV-1 encephalitis.

Materials and Methods

Mice, diets, and infection. Weanling BALB/cByJ male mice (Jackson Labs) were fed ad libitum 1 of 3 diets: 1) a VE-deficient (VE-D) diet (TD 88163), 2) a VE-A (dl-α-tocopheryl acetate) diet (38.4 mg/kg), or 3) a VE-supplemented (VE-S) diet (384 mg/kg) (Harlan Teklad) (Table 1). After 4 wk on the diets, the mice were lightly anesthetized with a ketamine (0.6 mg/kg) and xylazine (0.35 mg/kg) solution and infected i.n. with a 1.5 × 10^6 plaque forming unit (PFU) of HSV-1 in 10 μL total volume. All mice were housed 4 per cage in the University of North Carolina Animal Facility, which is fully accredited by the American Association for Accreditation of Laboratory Animal Care. Animals were maintained under protocols approved by the Institutional Animal Use and Care Committee.

HSV-1 virus stocks and virus inactivation. HSV-1 McIntyre (ATCC) stocks were propagated in Vero cells (ATCC), collected, centrifuged
(750 × g; 5 min), and stored at −80°C. Vero cells were maintained in DMEM supplemented with 2 mmol/L glutamine and adjusted with 1.5 g/L sodium bicarbonate, 0.1 mmol/L nonessential amino acids, 1.0 mmol/L sodium pyruvate, and 10% fetal bovine serum.

HSV-1 was inactivated by placing 1 mL aliquots in 30 mm tissue culture dishes (Becton-Dickinson) 2.5 cm from a germicidal UV light source for 6 min. Inactivation was confirmed by adding the inactivated virus to Vero cells to verify lack of viral replication.

Pathology and tissue collection. Following infection, mice were weighed, examined daily, and scored on the following scale: 0, no symptoms; 1, ruffled fur, ataxia; 2, hind-limb paralysis/forelimb claspimg; 3, hind-limb paralysis with forelimb weakness; 4, moribund; 5, dead. For PCR and viral titer experiments, uninfected (UNI, d0), d 3 and 7 postinfection (p.i.) mice were killed by rapid cervical dislocation, and the brain was removed and quickly dissected on ice and flash frozen.

Liver and brain α-TOC measurements. α-TOC levels were measured by HPLC following standard methods (35).

Brain cytokine measures. Levels of mRNA were determined by isolating total RNA from the forebrain region (thalamus and hypothalamus) using the TRIzol method (Invitrogen). Reverse transcription was carried out using Superscript II First Strand Synthesis kit (Invitrogen) with oligo (dT) primers. Expression of cytokine and chemokine mRNA was determined by quantitative RT-PCR (34). The levels of mRNA for glyceraldehyde-3-phosphate dehydrogenase were determined for all samples and used to normalize gene expression.

Brain stems (BS) were collected into 0.5 mL of ice-cold DMEM and homogenized, clarified by centrifugation (2500 × g; 3 min.), and stored at −80°C until assayed for regulated upon activation, normal T-cell expressed and secreted (RANTES) and IFN-γ inducible protein-10 with a Luminex-based multiplex ELISA kit (Biosource) and IL-1β and tumor necrosis factor (TNF-α ELISAs (BD Pharmingen) following the manufacturer’s instructions.

HSV-1 titers in brain. HSV-1 viral titers from the whole brain were determined from homogenized brain tissue by standard plaque assay on Vero cells. For viral titers from the olfactory bulb and brain stem genomic DNA, PCR was performed as previously described (36). DNA from UNI tissue was extracted in parallel and served as a negative control.

Statistics. Mortality data were analyzed by Kaplan-Meier survival analysis. All other data were analyzed by the nonparametric Kruskal-Wallis test. Kaplan-Meier survival analysis was performed using Prism 4 (GraphPad). All other statistical analyses were performed with JMP 6 software (SAS Institute). Values are the mean ± SEM. Data were considered statistically significant if P < 0.05.

Results

Brain and liver α-TOC levels. After 4 wk on the diet, mice were killed and their brains and livers removed. Altering α-tocopheryl acetate levels in the diets was effective in changing peripheral α-TOC levels. Liver levels of α-TOC were significantly decreased in VE-D mice compared with VE-A and VE-S mice (Fig. 1). The VE-S mice had nearly 7.5 times as much α-TOC as the VE-A mice. In the CNS, the temporal lobes of VE-D mice had significantly less α-TOC compared with the VE-A and VE-S mice (Fig. 1). However, in contrast to the liver, the high α-tocopheryl acetate diet was not effective in increasing brain α-TOC levels.

Mice on VE-D diets have earlier onset of HSE symptoms compared with mice on the VE-A diet. After 4 wk, mice were infected with 1.5 × 10⁶ PFU of HSV-1 and followed for symptoms of HSE. VE-D mice had increased HSE symptoms as well as a 28.6% mortality by d 7 p.i., whereas no VE-A mice died by d 7 p.i.

### Table 2

<table>
<thead>
<tr>
<th>Group</th>
<th>Mortality</th>
<th>Total symptom score</th>
</tr>
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<tbody>
<tr>
<td>VE-D</td>
<td>28.60</td>
<td>2.00 ± 0.44</td>
</tr>
<tr>
<td>VE-A</td>
<td>0.00</td>
<td>1.00 ± 0.26</td>
</tr>
<tr>
<td>VE-S</td>
<td>0.00</td>
<td>1.58 ± 0.45</td>
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1 Mice were examined daily for symptoms of HSE.
2 Percent survival through d 9 p.i. (P = 0.03); VE-D vs. VE-A, n = 14 mice/diet.
3 Values are means ± SEM; n = 6–10 mice (P < 0.01); VE-D vs. VE-A on d 7 p.i.

### Figure 3

Forebrain IFNβ (A), IFNγ (B), and iNOS (C) gene expression in VE-D, VE-A and VE-S mice. Data are the mean ± SEM, n = 6 or 7, and are expressed as the fold of the mean of the UNI VE-A group (d 0). *Different from VE-A at that time point, P < 0.05.
VE-A mice had fewer symptoms of encephalitis (and no mortality) compared with VE-D mice (Table 2). Following HSV-1 infection, VE deficiency increased cytokine and chemokine expression in the forebrain. Microglial cells are a key producer of proinflammatory and antiviral cytokines and chemokines in the brain during HSV infection (37). We examined the expression of cytokine and chemokines in the forebrain of VE-D, VE-A, and VE-S HSV-1 infected mice on d 0, 3, and 7 p.i. On d 7 p.i., the VE-D mice had significantly higher expressions of IL-6, TNFα, and IL-1β. IL-10 was significantly increased in VE-D mice on both d 3 and 7 p.i. (Fig. 2). As with the pathology scores, proinflammatory cytokine expression in VE-S mice did not differ from VE-A mice.

Gene expression for the antiviral cytokine IFNβ was significantly increased in VE-D mice compared with the VE-A mice on d 7 p.i. Gene expression of IFNγ was increased in VE-D mice on both d 3 and 7 p.i. Inducible nitric oxide synthase (iNOS) was significantly increased on d 7 p.i. in VE-D mice compared with VE-A mice, whereas VE-S mice had a decrease in iNOS on d 3 p.i. compared with VE-A mice (Fig. 3).

The expression of chemokines, which preceded the infiltration of large numbers of lymphocytes, and the upregulation of adhesion molecules were essential for T cells to enter the brain to clear HSV-1. Monocyte chemotactic protein-1 (MCP-1), RANTES, macrophage inflammatory protein-1α (MIP-1α), and intercellular adhesion molecule-1 were significantly higher on d 7 p.i. in the forebrain of VE-D mice compared with VE-A mice (Fig. 4).

VE deficiency increases proinflammatory cytokine and chemokine protein levels in the brain stem after HSV-1 infection. HSV-1 infected VE-A mice had an increase in protein levels for IL-1β, TNFα, and RANTES in the brain stem. VE-D mice had significantly more IL-1β and TNFα on d 7 p.i. Additionally, RANTES was significantly higher in the BS of VE-D mice on d 7 p.i. compared with VE-A mice (Fig. 5). Inducible protein-10 was increased with infection; however, no significant differences among the diet groups were found (data not shown). Similar to the mRNA levels in the forebrain, cytokine and chemokine production in the BS of VE-S mice did not differ from VE-A mice.

Increased HSV-1 viral load in VE-D mouse brains. HSV-1 viral replication in the brain leads to neuronal damage (38). To determine whether a deficiency in VE would lead to increased viral replication and, conversely, if increasing VE would lead to decreased viral replication, we measured HSV-1 titers in the brains of the mice. On d 7 p.i., both VE-D and VE-S mice showed a trend toward increased viral titers in the brain (Fig. 6A). The i.n. route of HSV-1 infection does not lead to the entire brain becoming infected, but rather HSV-1 spreads along the olfactory and trigeminal nerves to distinct regions (8); therefore, we examined individual regions of the brain for the presence of HSV-1. The HSV-1 genome was measured by DNA or mRNA in the olfactory bulb, brain stem (midbrain, pons, and medulla), and forebrain, respectively. On d 3 p.i., HSV-1 DNA was found in the olfactory bulb of all groups of mice, but it was not significantly higher in the VE-D or VE-S mice (Fig. 6B). However, the HSV-1 viral load was significantly higher in the brain stem and forebrain of VE-D mice on d 7 p.i. (Fig. 6C,D). No HSV-1 DNA or mRNA was detected in the brains of UNI mice.

**Discussion**

VE has been suggested as a treatment for HSV infections (39). However, there are few studies that have examined the effect of VE on HSV infections (40–42). Whereas supplementation with VE has become controversial (43,44), 93% of men and 96% of...
Nitric oxide may act as an immune mediator that leads to neuronal damage (48). iNOS, the enzyme that produces nitric oxide, is upregulated during HSV-1 infection. Its production plays a dual role in the response to HSV because iNOS is important for clearing infection (49), but too much is deleterious. iNOS is upregulated during HSV-1 infection in a temporal and spatial pattern that follows viral replication (50). iNOS inhibitors administered to mice infected i.n. with HSV-1 were demonstrated to significantly reduce paralysis and mortality (50). This suggests that iNOS plays a critical role in the pathogenesis of HSV-1 and that increased levels in VE-D mice may be a contributing factor that leads to the mortality in these mice.

Microglial cells are identified as a source of proinflammatory cytokine production during HSV-1 infection in both humans and mice (37,51,52). During HSV-1 infection, microglia from BALB/c mice produce a vigorous, but not protective response to HSV-1 (37). In the VE-D mice, the proinflammatory response was even more robust than in the VE-A mice. In light of the neurotoxic nature of these cytokines (53,54), it is likely that increased pathogenesis in VE-D mice is linked to this overly robust response.

Glutamate is released by microglia after activation by pro-inflammatory stimuli, including cytokines (55). An excessive glutamate release is neurotoxic, resulting in neuronal damage and neuroinflammation. In vitro, HSV-1–infected microglial cells release neurotoxic factors that result in neuronal death when the supernatants are transferred to neuronal cultures. The neurotoxic effects of these substances are partially blocked by iNOS inhibitors and N-methyl-D-aspartate (NMDA) receptor antagonists (54). Therefore, iNOS and glutamate-induced neurotoxicity via NMDA receptors may be partially responsible for HSV-1–associated neuronal damage. In vivo, administration of an NMDA-receptor antagonist to restraint-stressed HSV-1 mice decreases HSE pathology and mortality (19). In the brain, VE deficiency results in increased glutamate production (56). Together with the increase in glutamate, the high levels of proinflammatory cytokines produced in VE-D mice likely led to neurotoxicity, which might have been amplified by an increase in activated microglia.

Chemokines and adhesion molecules are upregulated in VE-A mice following the HSV-1 infection (10,37,57,58). This response was even more pronounced in VE-D mice. Chemokines and adhesion molecule expression are needed for T cells to cross the blood-brain barrier and enter the brain during HSV-1 infection (57,59). Future studies will examine the impact of increased chemokine and adhesion molecules on T-cell trafficking in VE-D mice. Additionally, the high α-TOC concentration in the periphery may alter T-cell function or trafficking in the VE-S mice.

CNS infection with HSV-1 results in oxidative stress and lipid peroxidation (60,61). Because VE-D alone increases oxidative stress and lipid peroxidation (22,62), and VE-D mice in this study had increased HSV-1 viral replication, it was not surprising that they had increased cytokine/chemokine production p.i. Previous studies demonstrate that VE is effective at controlling both peripheral and central inflammation, as well as reducing sickness behavior in LPS-treated mice (63–65). VE was considered a very good candidate for decreasing the symptoms of HSE. However, a 10× VE supplementation was unable to increase α-TOC levels in the brain over VE-A levels. Therefore, the lack of effect on cytokine and chemokine levels in the brains of VE-S mice was not unexpected. It is possible that a longer supplementation with 10× VE might increase brain α-TOC levels enough to be protective, this will require further study.

In addition to cytokine and chemokine production, symptoms of HSE are a result of the viral load in the various brain regions. Few studies examine the impact of antioxidant defi-
ciency on viral replication. Of the studies conducted, selenium deficiency results in increased coxsackievirus replication; however, it does not impact the replication of influenza A/PR8 (66,67). Both resveratrol, an antioxidant, and topically applied VE were shown to decrease HSV-1 replication (40,68). In this study, VE-D mice had a significantly higher viral load in the forebrain and brain stem compared with VE-A or VE-S mice. This is important because these regions are vital to maintaining whole-body homeostasis. The hypothalamus (part of the forebrain) is responsible for maintaining homeostasis by regulating thirst, hunger, circadian rhythms, and control of the autonomic nervous system. The brain stem controls breathing, heart rate, and blood pressure. High viral titers and inflammatory cytokines in these regions causing neuronal damage would be expected to result in the increased mortality seen in VE-D mice. The finding that VE-A and VE-S mice had similar titers is not a surprise given that $10^{3}$ supplementation was not effective in altering brain levels of α-TOC.

Taken together, these data indicate a global failure of VE-D mice to mount an appropriate immune response to a central HSV-1 infection and a failure of the $10^{3}$ VE-S to decrease HSE symptoms. These findings are important because the majority of people in the United States do not consume enough VE in their diets, suggesting that immune protection against HSV encephalitis, and perhaps other viral infections as well, may be suboptimal.

Acknowledgments
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Literature Cited
14. Ikemoto K, Pollard R, Fukumoto T, Morimatsu M, Suzuki F. Small amounts of exogenous IL-4 increase the severity of encephalitis induced

FIGURE 6 Brain HSV titer is expressed as means ± SEM, n = 5, of PFU per hemisphere of brain in VE-D, VE-A and VE-S mice (A). HSV-1 genomic DNA and mRNA in olfactory bulb (B), brain stem (C), and forebrain (D), respectively. Data are expressed as means ± SEM, n = 6 or 7. Abbreviations: N.Det., not detected; N.D., not done. *Different from VE-A at that time point, $P < 0.05.$


