The Role of the Blood-Brain Barrier during Venezuelan Equine Encephalitis Virus Infection

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Venezuelan equine encephalitis (VEE) virus is a mosquito-borne alphavirus associated with sporadic outbreaks in human and equid populations in the Western Hemisphere. After the bite of an infected mosquito, the virus initiates a biphasic disease: a peripheral phase with viral replication in lymphoid and myeloid tissues, followed by a neurotropic phase with infection of central nervous system (CNS) neurons, causing neuropathology and in some cases fatal encephalitis. The mechanisms allowing VEE virus to enter the CNS are currently poorly understood. Previous data have shown that the virus gains access to the CNS by infecting olfactory sensory neurons in the nasal mucosa of mice. However, at day 5 after inoculation, the infection of the brain is multifocal, indicating that virus particles are able to cross the blood-brain barrier (BBB). To better understand the role of the BBB during VEE virus infection, we used a well-characterized mouse model system. Using VEE virus replicon particles (VRP), we modeled the early events of neuroinvasion, showing that the replication of VRP in the nasal mucosa induced the opening of the BBB, allowing peripherally administered VRP to invade the brain. Peripheral VEE virus infection was characterized by a biphasic opening of the BBB. Further, inhibition of BBB opening resulted in a delayed viral neuroinvasion and pathogenesis. Overall, these results suggest that VEE virus initially enters the CNS through the olfactory pathways and initiates viral replication in the brain, which induces the opening of the BBB, allowing a second wave of invading virus from the periphery to enter the brain.

Venezuelan equine encephalitis (VEE) virus is an arthropod-borne single-stranded positive-sense RNA virus associated with periodic epidemics and epizootics in the Western Hemisphere. In equines, VEE virus causes a wide spectrum of disease ranging from unapparent illness to acute encephalitis (28). While enzootic strains cause minimal clinical illness in equines, disease induced by epizootic strains can be severe, causing high mortality (19% to 83%). In humans, the development of encephalitis appears in approximately 4% of cases with an overall case mortality rate of 1 to 2% (28).

Unlike the development of the disease in humans, inoculation of mice with a low dose of VEE virus into the rear footpad results in a rapidly progressing disease culminating in 100% mortality due to encephalitis. Virus replication in the draining lymph node (DLN) is detectable within 3 h. By 12 h, viremia is at or near its peak level; by 18 h, virus is replicating in virtually every lymphoid tissue, and a marked leukopenia is evident. Virus replication in the brain is detectable in olfactory tracts by 30 h postinfection (hpi), presumably having crossed from the circulation into olfactory sensory neurons (OSN) in the nasal olfactory neuroepithelium and hence along olfactory nerve tracts to the central nervous system (CNS). Encephalitis and death ensues 7 to 10 days after infection (3, 6, 11, 13, 21, 25).

Viral invasion and successful infection of the CNS is an important step in the life cycle of neurotropic viruses (14). The CNS is an immune-privileged site, protected by the blood-brain barrier (BBB) (5). The BBB is interposed between the circulatory system and the CNS and maintains the microenvironment of the brain (5, 18). The BBB is formed by brain capillary endothelial cells (BCEC), which line the cerebral microvessels. BCEC are specialized endothelial cells with tight junctions that make the lining of brain capillaries 50 to 100 times tighter than those in the periphery. The integrity of the BBB is maintained by the interaction of microvessel walls with perivascular endfeet of astrocytes and the continuous secretion of astrocytic-derived factors, such as transforming growth factor-β (TGF-β), glia-derived growth factor (GDNF), and basic fibroblast growth factor (bFGF) (1).

Pathogens have evolved several strategies that allow them to cross the BBB successfully and to gain access to the CNS. Herpes simplex virus and rabies virus spread transneurally from the site of entry and reach the CNS via axonal and/or retrograde transport. Other viruses cause viremia and use various mechanisms to cross the BBB and gain access to the CNS, including paracellular entry while the BBB is compromised, epithelium-mediated transcytosis, transmigration within infected leukocytes, or the infection of olfactory sensory neurons with subsequent axonal spread (22). In addition to these pathways, several host factors are associated with pathogen neuroinvasion: Toll-like receptor 3 (TLR-3), intercellular adhesion molecule 1 (ICAM-1), the matrix metalloproteinase 9 (MMP-9), interleukin-1β (IL-1β), and tumor necrosis factor alpha (TNF-α) promote the ability of West Nile virus (WNV) to cross the BBB (7, 26, 27).

For VEE virus, the olfactory neuroepithelium has been suggested as a primary entryway into the CNS (6, 21). In previous studies, viral replication was detectable at 24 to 30 h in OSN of the olfactory bulbs (OB) after footpad inoculation. At 30 to 36
were restrained and 2

All experimental manipulations of mice with VRP were performed under BSL-2

weight loss. Mice that lost more than 20% of their starting body weight or

animals were monitored and assessed daily for development of disease signs and

when used for infection studies. After mice were anesthetized with a ketamine-

acclimatization period, such that mice were approximately 6 to 10 weeks old

performed in a biosafety level 3 (BSL-3) animal facility following at least a 7-day

mined by indirect immunofluorescence in BHK-21 cells using a polyclonal anti-

passage of a sample on BHK-21 cells and examination of cytopathic effect (CPE),

linearized cDNA clone using a T7-specific mMessage mMachine kit (Ambion)

Virus titers. To quantitatively V3000 titers in vivo, infected or mock-infected ani-
mals were deeply anesthetized, bled, and perfused with PBS as previously de-
scribed (4). After euthanasia, tissues and serum were collected and frozen at

RESULTS

VRP replication in nasal mucosa induces opening of the
BBB. Previous studies have shown that VEE virus is able to
infect and replicate in the murine nasal neuropil (6). To
confirm this observation and determine the BBB integrity
during VEE virus infection of the nasal mucosa, mice were
inoculated i.n. with 2 × 10⁶ IU VRP or with diluent alone
and assayed for BBB permeability 24, 48, and 96 hpi. One
hour prior to harvest, the animals were inoculated i.p. with 800 µl
1% Evans blue dye. The results showed minimal staining at 24
hpi. However, at 48 hpi after i.n. VRP inoculation, a significant
opening of the BBB throughout the brain was observed (Fig. 1),
which was also confirmed with immunofluorescence studies
(data not shown). In addition, a strong preferential staining of
the frontal lobe in the area where the olfactory cortex is
located (indicated by arrows) was observed at this time point.
No staining of other areas was detectable at 96 hpi. Control
animals that received i.n. diluent alone showed no signs of a
compromised BBB (Fig. 1). These data demonstrate that VRP
infection in the nasal neuropil induces a breakdown of the
BBB, detectable by day 2 post-i.n. inoculation, with an
intensified staining pattern in the frontal lobe.

Intranasal inoculated VRP replicate in the nasal mucosa
but do spread into the CNS. To follow VRP RNA replication
in the CNS after intranasal inoculation, total RNA was har-
vested from the olfactory bulbs, the cortex, and the cerebellum/pons at 6, 12, 24, 48, and 72 hpi. After cDNA synthesis, viral plus- and minus-strand RNA was quantified by real-time PCR, and as shown in Fig. 2, no viral RNA replication was detectable in the cortex or the cerebellum/pons at any of the indicated time points. In the olfactory bulb, viral RNA replication peaked at 12 to 24 hpi and dropped to below the detection level by 96 hpi. Taking these findings together with previous data that identified olfactory sensory neurons within the nasal mucosa as the main target cells for VEE virus infection and replication (6), it can be suggested, that (i) VRP replicate in the nasal mucosa and (ii) VRP are able to reach the outer layers of the olfactory bulb through the axons of actively infected olfactory sensory neurons, but (iii) VRP do not spread across the synapse connecting the OSN with second-order CNS neurons within the olfactory bulb (Fig. 2).

Intranasal VRP inoculation induces the expression of proinflammatory genes in different parts of the CNS. The upregulation of certain proinflammatory cytokines and chemokines mediates the breakdown of the BBB (23). To analyze the induction of proinflammatory genes in the CNS by VRP infection, mice were inoculated i.n. with $2 \times 10^6$ IU VRP. At 6, 12, 24, 48, and 96 hpi, brains were harvested and dissected for total RNA isolation. The expression of IFN-β, TNF-α, and IL-6 (Fig. 3A to C) was detectable 12 to 24 hpi in the olfactory bulbs. At 24 to 48 hpi, the expression of those genes reached a maximum level in the cortex. The expression levels of all 3 genes were waning by 96 hpi. MMP-9 has an important role in maintaining the integrity of the BBB (2, 20), and several studies have described a role of MMP-9 in pathogenesis during viral CNS infections (15, 26, 31). As early as 12 hpi following VRP infection, increased MMP-9 expression was detectable in the whole brain; by 48 hpi, expression reached maximal levels in the cortex, while declining in the olfactory bulbs and the cerebellum/pons (Fig. 3D). A pattern similar to that of MMP-9 was observed for ICAM-1 expression, a molecular marker for BBB breakdown (23), with detectable expression by 12 hpi and 24 hpi in the whole brain and with peak expression by 48 hpi in the cortex (Fig. 3E). Slightly elevated expression levels were observed in the cerebellum/pons in a temporal pattern similar to that in the olfactory bulbs. This could be explained by the fact that the nasal mucosa is innervated by the trigeminal nerve.

These data indicate that VRP replication in the nasal mucosa induces the expression of proinflammatory genes in the brain, as well as MMP-9 and ICAM-1, without VRP or VRP genomes reaching the brain (16, 17, 19, 23). The expression levels showed a temporal and spatial pattern, with maximal expression early in the OB, followed by increased expression in the cortex and the cerebellum/pons at later time points. The observations suggest that cytokine signals, induced initially by VRP infection of the olfactory sensory neurons, act in a paracrine manner on adjacent neurons in the OB and olfactory tract. These signals, in turn, induce the target neurons to produce a cytokine response that operates on the next neuron as well as other bystander cells. Ultimately, propagation of such cytokine signals results in an early spatial pattern of BBB opening.
10^6 IU VRP or diluent alone. At 24 or 48 hpi, both test and control mice were inoculated i.v. with RFP-VRP or with diluent alone. After an additional 24 or 48 h, mice were euthanized and brains harvested and prepared as frozen sections (Fig. 4A). Only in samples primed with VRP i.n. was RFP expression detectable in the brains at 48 hpi after intravenous RFP-VRP injection (Fig. 4B). RFP expression was not detectable in samples where the mice were initially inoculated i.n. with diluent alone.

To exclude the possibility that the RFP-VRP proteins were “cocarried” into the CNS by infected peripheral lymphocytes, sections were costained for NeuN. As shown in Fig. 4B, all RFP-expressing cells were identified as CNS neurons by positive staining for NeuN (23). This result required that RFP-VRP cross the BBB as intact infectious virus particles and then infect CNS neurons to allow detection of RFP expression. Therefore, these data strongly suggest (i) that the opening of the BBB induced by viral replication in the nasal mucosa is sufficient to allow viral neuroinvasion from the periphery and

![Graphs of IFN-β, TNF-α, IL-6, MMP-9, and ICAM-1 expression](image)

**Fig. 3.** Proinflammatory genes were expressed in different anatomical parts of the brain after i.n. VRP inoculation. Mice were inoculated with 2 \times 10^6 IU VRP or diluent alone, and at 6, 12, 24, 48, and 96 hpi mice were sacrificed and their brains were harvested and dissected into olfactory bulb, cortex, and cerebellum/pons for total RNA isolation. cDNA was synthesized, and expression levels for IFN-β, TNF-α, IL-6, MMP-9, and ICAM-1 were determined by quantitative real-time PCR, normalized to GAPDH values, and analyzed in comparison to mock-infected tissue. Data presented are the means ± standard errors of the means for four mice per group.

![Graph of RFP-VRP distribution](image)

**Fig. 4.** RFP-VRP inoculated intravenously were detectable in the brain after VRP-induced opening of the BBB. Mice were inoculated i.n. with 2 \times 10^6 IU VRP or diluent alone. After 24 and 48 h, i.n. VRP-inoculated mice were inoculated with 2 \times 10^6 IU RFP-VRP intravenously. (A) At 24 and 48 h post-i.v. injection of RFP-VRP, mice were sacrificed and brains were harvested for cryoprotection. (B) After sectioning tissues at 10 μm sagitally, sections were stained for NeuN expression and were examined by fluorescence microscopy.
that CNS invasion by circulating virus can occur through an open BBB.

**V3000 infection opens the BBB.** Previous studies have suggested the involvement of the BBB in viral CNS infections. To evaluate the status of BBB integrity during a peripheral VEE wild-type virus infection, mice were inoculated with $10^3$ PFU V3000, or with diluent alone as a control, into the left rear footpad (Fig. 5). The infected animals were then monitored for weight loss and disease development, and brains were harvested daily from 1 to 7 dpi to assess BBB integrity. As shown in Fig. 5, an increase of Evans blue dye uptake was first detectable at 3 dpi. The brains were less intensely stained on 4 and 5 dpi, but by 6 dpi a second peak of BBB breakdown was observed. The data indicated that the BBB permeability changes during the course of V3000 infection and the opening of the BBB shows a biphasic pattern, with maximal peaks at 3 and 6 dpi. Temporally, the first phase may correlate with prior virus replication in the nasal neuroepithelium and the second phase may result from invasion of and replication in the CNS.

**Proinflammatory genes are expressed during peripheral V3000 infection.** A robust and rapid cytokine response has been described during V3000 infection (12). The CNS is able to increase cytokine expression as early as 6 to 12 h after peripheral VRP infection in the footpad, presumably mediated by circulating cytokines and under conditions where neither VRP nor VRP genomes are found in the CNS (17, 29). Even though this response is thought to set the CNS into an alerted state, peripheral VRP replication did not cause any detectable effect on BBB integrity (23). To study the expression levels of TNF-α, IL-6, MMP-9, and ICAM-1 (Fig. 6A to D), which were thought to be involved in BBB opening, mice were peripherally inoculated with $10^3$ PFU V3000 and both serum and brains of the infected animals were harvested for total RNA isolation for gene expression analysis at 1 to 7 dpi. Early in the infection, from 1 to 4 dpi, the expression levels of both TNF-α and IL-6 were elevated in the serum of V3000-infected mice. By 6 dpi levels in the serum started to decline, while expression levels in the brain increased and remained at a plateau until the death of the animals. These expression patterns differed from the pattern observed for MMP-9. MMP-9 levels also increased in the serum of the infected animals for the first 2 days of infection, but at 3 dpi MMP-9 levels started to rise in the brain. A second peak of expression was detectable at 6 dpi. During this late phase of infection, expression levels of MMP-9 remained 5 to 10 times higher in the brain than in the serum.

As described previously, ICAM-1 has an important role in attachment of leukocytes to endothelial cells, and its expression is induced by TNF-α, IL-1β, and type I IFN (10). A quantification of ICAM-1 expression in brains of V3000-infected mice showed low levels on 1 and 2 dpi; by 3 dpi expression reached its maximum and stayed level, reflecting 10 to 15 times higher expression on 4 to 7 dpi compared to control animals. The *in vivo* expression and localization of MMP-9 in the brain of mice inoculated i.n. with $2 \times 10^6$ IU VRP was studied by immunofluorescence (Fig. 6E). A strong signal for MMP-9 expression was specifically detectable in and around the blood vessel walls throughout the brain, suggesting that the MMP-9 expression was localized within the brain.

Taken together, these data demonstrate that expression of TNF-α and IL-6 was induced early in the periphery during virus infection. Expression levels increased slowly during the early phase of virus infection but increased significantly after the virus reached and infected the CNS, i.e., the neurotropic phase of virus infection. The pattern observed for MMP-9 expression during V3000 infection was consistent with induction from inside the CNS, triggered by virus replication starting by 3 dpi.
Inhibition of opening of the BBB delays disease onset. To study the effect of a closed BBB on the course of V3000 peripheral infection, we inhibited the breakdown of the BBB by using the MMP-9 inhibitor GM6001 (Ilomostat). Groups of mice were treated with the inhibitor 3 h prior to infection and at 1 and 3 dpi. At 3 and 6 dpi, treated and untreated animals were inoculated with Evans blue dye to evaluate BBB integrity after treatment. No BBB breakdown was detectable in treated mice at 3 dpi compared to untreated mice (Fig. 7A). At 6 dpi, a partial inhibition of BBB breakdown in treated mice was observed compared to untreated mice (Fig. 7A).

To evaluate the progression of disease development and average survival time, groups of mice were treated with the inhibitor (n = 24; dotted line), infected, and compared to infected but untreated control mice (n = 8; dashed line). Infected untreated mice started to show signs of developing encephalitis by 3 dpi (data not shown) (4, 17), and all infected but untreated animals succumbed to viral infection between 5 and 8 dpi (Fig. 7B). Infected mice that were treated with the inhibitor showed an average survival time of 10 days, and by 14 dpi, 17% of the treated animals were still alive (Fig. 7B).

To ensure that the delayed onset of disease and increased survival time was due to an inhibition of the opening of the BBB, treated (n = 6; dotted line) and untreated (n = 4; dashed line) groups of mice were inoculated with 1 x 10^6 PFU V3000 intracranially (i.c.) to bypass the BBB. As shown in Fig. 7C, no...
significant differences were observed between test and control groups: treated and untreated animals showed the same course of disease development and an average survival time of 3 to 4 days with a 100% mortality rate.

Taken together, these data indicate that the inhibition of the BBB opening during a peripheral V3000 infection prolonged the survival of infected mice that did succumb by 2 to 3 days, with a 17% survival rate at 14 dpi, compared to 100% mortality in untreated animals.

Inhibition of the opening of the BBB delayed viral neuroinvasion. To inhibit the opening of the BBB during a peripheral V3000 infection, mice were treated with GM6001 3 h prior to V3000 infection, and at 1 and 3 dpi, mice were infected with $10^2$ PFU V3000 via injection into the rear footpad. At 3 and 6 dpi, V3000-infected animals were treated with or without GM6001 were inoculated i.p. with 800 µl 1% Evans blue dye. One hour after Evans blue dye injection, mice were sacrificed and brains were harvested. After infection, treated and untreated animals were monitored daily for determination of survival time. To bypass the BBB while under GM6001 treatment, mice were infected i.c. with $10^2$ PFU of V3000 and monitored daily for disease development and survival rate. Red arrows indicate time points of GM6001 administration. *, $P < 0.05$ by log-rank (Mantel-Cox) test.

FIG. 7. Inhibition of the opening of the BBB in V3000-infected mice delayed disease onset. Mice were treated with GM6001 3 h prior to V3000 infection, and at 1 and 3 dpi, mice were infected with $10^2$ PFU V3000 via injection into the rear footpad. (A) At 3 and 6 dpi, V3000-infected animals treated with or without GM6001 were inoculated i.p. with 800 µl 1% Evans blue dye. One hour after Evans blue dye injection, mice were sacrificed and brains were harvested. (B) After infection, treated and untreated animals were monitored daily for determination of survival time. (C) To bypass the BBB while under GM6001 treatment, mice were infected i.c. with $10^2$ PFU of V3000 and monitored daily for disease development and survival rate. Red arrows indicate time points of GM6001 administration. *, $P < 0.05$ by log-rank (Mantel-Cox) test.
DISCUSSION

The invasion of the CNS is an important step in the pathogenesis of neurotropic viruses. Many CNS-infecting viruses first establish infection in the periphery before successfully entering the brain, but the underlying details and exact pathways of neuroinvasion remain an active research interest (14, 22). Based on previous studies, we developed the hypothesis that VEE virus initially enters the brain by developing a high-titer viremia, followed by the infection of olfactory sensory neurons, thereby gaining access to the olfactory bulbs. Then, viral replication in the CNS induces the opening of the BBB, which allows a second wave of invading virus from the periphery to enter the CNS. Taking advantage of a well-established mouse model system for VEE virus infection and pathogenesis (3, 4, 13, 25) as well as the VRP system (16, 19, 23), we were able to dissect the steps of VEE virus neuroinvasion and to describe an important role of the BBB in VEE virus CNS infection.

To model the early events of VEE virus neuroinvasion, we took advantage of the well-characterized VRP system (16, 19, 23). VRP infect cells normally, undergo RNA replication, and express marker genes, but their genomes lack the genes for the structural proteins. Thus, they are unable to assemble new virus particles and spread beyond the initially infected cell. Infection with VRP allows us to directly assess possible effects on the integrity of the BBB in the absence of virus spread to or infection of the brain.

The first consideration in examining this hypothesis is that VRP replicate in the nasal mucosa after i.n. inoculation without spreading to the CNS. This was confirmed by the findings that neither plus- nor minus-strand RNA is detected in the CNS by PCR after i.n. VRP infection (Fig. 2).

Second, the temporal pattern of cytokine induction is consistent with propagation of a cytokine signal along the olfactory nerve (Fig. 3). We showed previously that VRP-infected cells can induce a bystander cytokine response in a paracrine manner (16, 23). In this study we showed that infected OSN in the nasal mucosa send signals into the brain, where bystander cells initiate the production of cytokines, leading to a propagating bystander response along the olfactory nerve. At this point, we can only speculate about the composition of bystander cells, which could be CNS neurons, astrocytes, and microglia cells or a combination thereof. Cytokine induction is observed first in the olfactory bulb and later in the cortex in the absence of viral replication at that site (Fig. 2 and 3).

Third, gene expression profiles of markers associated with BBB opening, like ICAM-1, and inducers of BBB opening, like TNF-α and MMP-9, are upregulated in the olfactory bulb and cortex by i.n. infection with VRP (Fig. 3). In addition, the temporal pattern of expression is consistent with propagation of a bystander cytokine signal along the olfactory nerve.

Fourth, the spatial and temporal aspects of BBB opening are consistent with our hypothesis. In the brain, focal opening of the BBB was first observed in the cortical areas associated with olfactory tracts, followed by a more general opening of the BBB throughout the brain (Fig. 1). This observation is consistent with an initial cytokine signal reaching the brain along the olfactory nerve.

Therefore, the observation by Charles et al. (6) that an in situ hybridization signal in the brain was first detected in olfactory tracts is consistent with (i) transsynaptic spread of virus along the olfactory nerve from the nose to the CNS as well as (ii) transsynaptic signaling along the olfactory nerve leading to focal opening of the BBB and consequent invasion of the brain at that site by virus from the circulation. The latter was demonstrated by infecting mice with VRP i.n. and then demonstrating infection of neurons in the brain after i.v. inoculation of RFP-VRP (Fig. 4). In the absence of a previous i.n. infection, which would induce opening of the BBB, RFP-VRP did not cross into the brain. These data also show that RFP-VRP invasion of the brain was not due to the entry of VRP-infected cells from the periphery, nor was an infection of endothelial cells by VRP observed.

Finally, cytokine signaling along the olfactory nerve seems highly relevant to VEE disease, as inhibitor studies suggest that blocking the opening of the BBB delays neuroinvasion and in some instances prevents the development of lethal disease (Fig. 7 and 8).

To further investigate the involvement of the BBB during neuroinvasion, we studied its role during peripheral (footpad) VEE wild-type virus infection. BBB integrity was compromised during infection and showed maximal opening at 3 dpi, the time point when the viral load in the CNS first increases (Fig. 5); viremia was still at a high level then and at 6 dpi, the time when cells of the adaptive immune system enter the infected brain (4).

Previous studies have shown that the inhibition of MMP-9 activity prevents the breakdown of the BBB and severe pathology in West Nile virus (WNV) infection (26). MMP-9 has been described to have an important role in maintaining BBB integrity and to be involved in neuroinvasions by WNV (20, 26). In this study, there is a significant upregulation of MMP-9 in the brain after intranasal VRP inoculation and early during peripheral VEE virus infection (Fig. 3 and 6). Later in infection, entering neutrophils from the periphery are also potential alternative sources of activated membrane-associated MMP-9 (30). In any event, MMP-9 expression was specifically detectable by immunofluorescence staining at 2 dpi in endothelial cells around brain blood vessels (Fig. 6E), indicating the activity of this enzyme within the brain was most likely responsible for BBB opening. The treatment of VEE virus-infected mice with the MMP-9 inhibitor prevented the opening of BBB at 3 dpi, and BBB opening was significantly diminished at 6 dpi (Fig. 7). Importantly, a significant delay in onset of disease was observed in animals treated with the MMP-9 inhibitor, which was correlated with a significant delay in neuroinvasion (Fig. 8), resulting in an increase of the average survival time by 2 to 3 days (Fig. 7).

Several additional points of interest flow from this work. First, inhibition of BBB opening during the first stages of infection resulted in an increase in average survival time by 2 to 3 days, and decreased mortality. It is interesting to speculate whether a more dramatic increase in survival may have resulted from long-term treatment with the MMP-9 inhibitor.

Second, neuroinvasion during VEE virus infection could occur by one or more of three mechanisms: (i) direct progression across synapses from the OSN to olfactory CNS neurons, (ii) transmission of a cytokine signal to CNS olfactory tracts resulting in local opening of the BBB and consequent local invasion of the CNS from the circulation, and/or (iii) global
opening of the BBB after initial CNS infection followed by entry of circulating virus. The MMP-9 inhibitor theoretically should not have affected transsynaptic virus transmission from OSN along the olfactory nerve to the CNS. Detailed examination of this pathway in the presence of the inhibitor will determine whether virus or only the cytokine signal is transmitted.

Third, inhibition of BBB opening is likely to reduce entry of cells involved in the adaptive immune response. This could result in both a reduction in the beneficial effects of the immune response or in potentially immunopathogenic effects, thereby complicating the interpretation of experiments with the MMP-9 inhibitor.

Fourth, is the initial invasion of the brain sufficient for mortality? Clearly, inhibition of BBB opening early in infection reduces mortality and morbidity, but ascribing this effect to a specific event must await definition of early and late neuroinvasion and experimental differentiation between them. However, differences in degree and timing of BBB opening could account for species-specific differences in susceptibility to lethal VEE virus encephalitis. For instance, encephalitis occurs in 100% of mice, 30 to 70% of equines, and ~4% of humans.

Finally, although we cannot assume that the neuroinvasion mechanism defined here may apply to VEE virus infection in humans, it may be possible to utilize intranasal infection with VRP to transiently open the BBB for treatment of CNS disease or otherwise-un treatable tumors. Antibodies and approximately 98% of small-molecule drugs are prevented from entering the CNS due to the BBB, thus complicating and severely reducing treatment options for life-threatening infections and tumors of the CNS (8, 9).

In summary, we have presented a modified hypothesis regarding neuroinvasion and neuropathogenesis during VEE virus infection of the mouse. The first neurons infected are OSN, where unmethylated nerves and fenestrated capillaries in the nose are anatomically juxtaposed, allowing neuronal infection by virus circulating in the blood. Viral replication in OSN induces a cytokine signal that is transmitted across synapses to olfactory tracts in the brain, causing local opening of the BBB and local invasion from the circulation directly into the CNS. Alternatively, or in addition, virus itself moves up the olfactory nerve to the CNS. Either or both pathways result in an initial seeding of the CNS, amplified cytokine signaling, widespread breakdown of the BBB, and consequent invasion of circulating virus. Blocking the opening of the BBB early after subcutaneous infection could restrict initial local seeding of the brain, the more widespread second phase of neuroinvasion, and the resulting morbidity and mortality characteristic of VEE virus infection.

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