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REVIEW

Alphavirus Encephalomyelitis: Mechanisms and Approaches to Prevention of Neuronal Damage

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Abstract Mosquito-borne viruses are important causes of death and long-term neurologic disability due to encephalomyelitis. Studies of mice infected with the alphavirus Sindbis virus have shown that outcome is dependent on the age and genetic background of the mouse and virulence of the infecting virus. Age-dependent susceptibility reflects the acquisition by neurons of resistance to virus replication and virus-induced cell death with maturation. In mature mice, the populations of neurons most susceptible to infection are in the hippocampus and anterior horn of the spinal cord. Hippocampal infection leads to long-term memory deficits in mice that survive, while motor neuron infection can lead to paralysis and death. Neuronal death is immune-mediated, rather than a direct consequence of virus infection, and associated with entry and differentiation of pathogenic T helper 17 cells in the nervous system. To modulate glutamate excitotoxicity, mice were treated with an *N*-methyl-D-aspartate receptor antagonist, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor antagonists or a glutamine antagonist. The *N*-methyl-D-aspartate receptor antagonist MK-801 protected hippocampal neurons but not motor neurons, and mice still became paralyzed and died. α-Amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor antagonists GYKI-52466 and talampanel protected both hippocampal and motor neurons and prevented paralysis and death. Glutamine antagonist 6-diazo-5-l-norleucine protected hippocampal neurons and improved memory generation in mice surviving infection with an avirulent virus. Surprisingly, in all cases protection was associated with inhibition of the antiviral immune response, reduced entry of inflammatory cells into the central nervous system, and delayed virus clearance, emphasizing the importance of treatment approaches that include prevention of immunopathologic damage.

Keywords Glutamate excitotoxicity · Immunopathogenesis · Sindbis virus · AMPA receptor antagonist · Glutamine antagonist

Introduction

Viral encephalomyelitis can be a devastating disease for the infected individual and for society as a whole because those who recover are frequently left with neurological sequelae such as seizures, paralysis, and cognitive deficits [1]. Arthropod-borne (arbo) viruses are important causes of encephalomyelitis with widespread seasonal outbreaks of fever, encephalitis, and arthritis, and pose increasing threats to human populations through continued expansion into new geographic areas [2, 3]. Alphaviruses are mosquito-borne plus-strand-enveloped RNA viruses that cause both encephalomyelitis (Venezuelan, western and eastern equine encephalitis viruses) and arthritis (Sindbis, Ross River, and Chikungunya viruses). The encephalitic alphaviruses are endemic in the Americas, while the rapidly emerging arthritic alphaviruses that can also cause neurologic disease are now found worldwide [2, 4–7]. Currently,

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there are no treatments or vaccines licensed for these infections.

For all arboviruses, severity of disease and outcome after infection varies widely from one person to another. This review will cover our current knowledge of the pathogenesis of fatal encephalomyelitis, the determinants of variable outcome, and potential treatments for severe disease through the study of central nervous system (CNS) infection by the prototype alphavirus, Sindbis virus (SINV), in mice.

Neuronal Damage

Neurons are the primary target cells of encephalitic alphaviruses and the outcome of infection is determined both by neuronal maturity and infecting virus virulence. Susceptibility to fatal encephalomyelitis diminishes with increasing age [8], and this is not due to changes in the adaptive immune response, but rather to changes in the intrinsic susceptibility of immature and mature neurons to infection [9]. Neuroadapted strains of SINV (e.g., NSV) with improved replication in mature neurons can cause fatal encephalomyelitis in older mice, providing a model system for evaluation of therapeutic interventions. Populations of mature neurons that are particularly susceptible to infection are in the hippocampus and anterior horn of the spinal cord. NSV-infected motor neurons die by a nonapoptotic process [10], and mice develop weakness that progresses to paralysis and death within 7–10 days [11, 12].

Role of Neuronal Maturity

Immature neurons of all types replicate SINV (and other neurotropic arboviruses) to high titers that result in death due to apoptosis, while mature neurons restrict virus replication, are relatively resistant to virus-induced apoptosis, and can become persistently infected (Fig. 1a) [13–15]. Maturation-dependent restriction of virus replication is also observed in cultured primary neurons (e.g., dorsal root ganglia cells) and neuronal cell lines differentiated *in vitro* (e.g., CSM14.1, AP-7) (Fig. 1b), facilitating mechanistic studies [13, 16–18].

Neuronal maturation in the absence of infection is associated with increased expression of interferon (IFN)-β, transcription factors IFN regulatory factor (IRF)-3 and IRF-7, and several IFN-stimulated gene (ISG) mRNAs (e.g., 2,5OAS, RNaseL, β2 m, IFIT1, IFIT3, ISG20) [15]. IRF-7 is a key transcription factor, with multiple splice variants, that regulates and amplifies the IFN response through induction of the IFN-α genes, as well as ISGs [19]. The IRF-7 protein

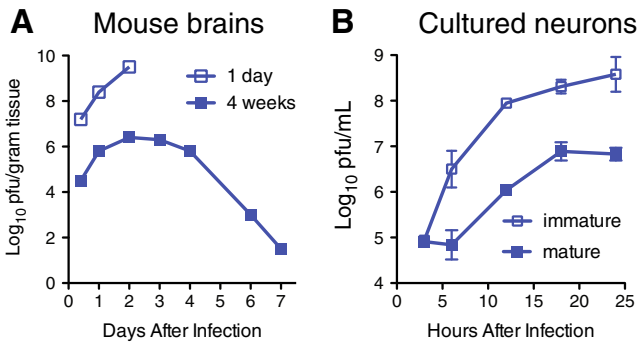

Fig. 1 Neuronal maturation leads to restriction of Sindbis virus replication. **a** Virus replication in the brains of 1-day and 4-week-old mice after intracerebral inoculation [8]. **b** Virus replication in immature undifferentiated and mature differentiated AP-7 rat neuronal cells [15]

produced by neurons evolves with maturation from the short dominant negative γ isoform to the full-length active α isoform necessary for transcribing antiviral protein genes [15]. With maturation, uninfected neurons produce small amounts of IFN-β that results in priming the cell for an antiviral response. Neutralization of IFN increases replication of SINV, suggesting that the low levels of IFN constitutively produced by mature neurons are important for resistance [16]. In response to infection, differentiated, but not undifferentiated, neurons rapidly produce IFN and upregulate ISGs to restrict virus replication. Therefore, neuronal maturation is associated with antiviral priming characterized by increased basal levels of important transcription factors that rapidly activate antiviral signaling in response to infection, and thus reduce virus replication in mature neurons [15].

Role of Virus Strain

Alphaviruses have a message-sense RNA genome that encodes 4 nonstructural replication proteins (nsP1–4), 3 main structural proteins (capsid and envelope proteins E1 and E2), and 2 small proteins (6 K, TF). NSV is a neuroadapted strain of SINV that is virulent for adult C57BL/6 mice and provides a model for developing an understanding of virus-induced fatal encephalomyelitis in mature animals [20]. NSV has the same neuronal tropism as less virulent strains of SINV but replicates to higher titer and induces more intense inflammation in the brain and spinal cord [11, 21].

Virulence determinants are primarily in the E1 and E2 glycoproteins that regulate virus entry into neurons, alter glycosylation, and change binding to heparan sulfate [22–27]. In addition, recent studies have identified important roles for changes in nsP3, TF, and the 5' nontranslated region that influence neuronal replication and alter virulence [28–30].

Role of the Immune Response

Virus clearance from neurons is accomplished through a synergistic process involving T-cell production of IFN-γ and B-cell production of antibody to the E2 glycoprotein [13, 31, 32]. Therefore, in response to infection, T-cell-mediated inflammation and B-cell infiltration into the CNS are necessary for virus clearance but need to be regulated to prevent damage to neural tissue [33].

Several observations have led to the conclusion that neuronal damage in mature animals is primarily due to the antiviral immune response rather than virus replication per se, and that fatal alphaviral encephalomyelitis is a T-cell-mediated immunopathologic process. For instance, initiation of virus clearance and the inflammatory response are coincident with the onset of neurological disease [21], and survival is improved in mice deficient in αβ T cells, β2-microglobulin, transporter associated with antigen processing (TAP), or CD4 but not in mice deficient in production of antibody, CD8, perforin, Fas, TNF-α receptor-1, IFN-γ, IFN-γ receptor-1, or IL-6 [34–36]. Furthermore, mice protected from fatal disease by passive transfer of immune serum after NSV infection clear infectious virus but develop a progressive loss of parenchyma (*ex vacuo* hydrocephalus) associated with infiltration of $CD4^+$ T cells and macrophages into the hippocampus [35].

IL-10 is an important regulatory cytokine that helps to determine the balance between inflammation and immunoregulation [37, 38]. Deficiency of IL-10 accelerates the onset of fatal NSV-induced paralytic disease with an early increase in the CNS of $CD4^+$ T cells expressing the transcription factor RORγt and producing the cytokine IL-17 [T helper (Th) 17 cells] [39]. Th17 cells are multifunctional and can have pathogenic or nonpathogenic characteristics. In response to NSV infection, Th17 cells in the CNS (but not in the draining lymph nodes) had a pathogenic phenotype with production of granulocyte macrophage colony-stimulating factor (GM-CSF) and granzyme B. In addition, some Th17 cells in the CNS developed into doubly pathogenic Th1/Th17 cells with additional expression of the transcription factor T-bet and production of IFN-γ. Although pathogenic Th17 cells are recognized to be effectors in autoimmune disease [40], they were not previously identified as contributors to virus-induced immunopathology [41].

These studies and comparative studies of BALB/c mice that are genetically resistant to fatal NSV-induced encephalomyelitis indicate the importance of IL-10 in regulating the immunopathogenic effects of antiviral T cells [42]. $CD4^+$ T cells infiltrating the brains of BALB/c mice include fewer Th17 cells and more regulatory T cells producing IL-10 than similarly infected C57BL/6 mice [42]. In the absence of IL-10 BALB/c mice become susceptible to fatal infection. The primary sources of regulatory IL-10 during infection are the infiltrating $CD4^+$ and $CD8^+$ lymphocyte populations, not myeloid cells intrinsic to the CNS [43].

Determination of the role of Th17 cells in NSV-induced immunopathology and identification of the mechanism(s) by which they influence outcome will be important for developing interventions and for identifying host determinants of susceptibility to severe disease. Th17 cells can directly target neurons [44], and under conditions of stress *in vitro*, neurons express IL-17 receptor and treatment with IL-17 can induce neuronal cell death [45]. GM-CSF has also been identified as a potential mediator of neural damage [46–49]. GM-CSF activates microglial cells and recruits myeloid cells into the CNS, but the mechanism by which this leads to disease has not been identified [47, 50, 51]. Furthermore, neutralizing antibody to neither GM-CSF nor IL-17 altered the course of disease compared with control antibody in either $IL\text{-}10^{-/-}$ or wild-type mice [39].

Prevention of Fatal Disease

Our studies indicate that development of successful treatments for viral encephalomyelitis requires a strategy to decrease immunopathologic damage either as a primary approach or as an adjunct to use of an antiviral drug. Damage to hippocampal and motor neurons suggested that glutamate excitotoxicity might play a role in inducing neuronal death. During infection, excess glutamate may result from extracellular release by damaged neurons or microglial cells [52], production by activated $CD8^+$ T cells entering the CNS [53], or from failure of astrocytes to remove excess glutamate due to a cytokine-induced decrease in expression of glutamate transporter-1 [54, 55]. *In vitro* treatment of infected primary cortical neurons with *N*-methyl-D-aspartate receptor antagonists MK-801 and D(-)-2-amino-5-phosphonopentanoic acid (APV) decreased cell death [56]. Although treatment of NSV-infected mice with MK-801 protected hippocampal neurons, it did not protect motor neurons or prevent paralysis and death of the mice (Fig. 2a) [57]. However, treatment of NSV-infected mice with α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor antagonists GYKI-52466 or talampanel protected both hippocampal and motor neurons, improved outcome, and indicated that fatal disease is primarily due to infection of motor neurons rather than hippocampal neurons (Fig. 2a) [57, 58]. Surprisingly, upon examination of the mechanism of protection, it was discovered that AMPA receptor antagonists actually suppressed the antiviral immune response and subsequent entry of inflammatory cells into the

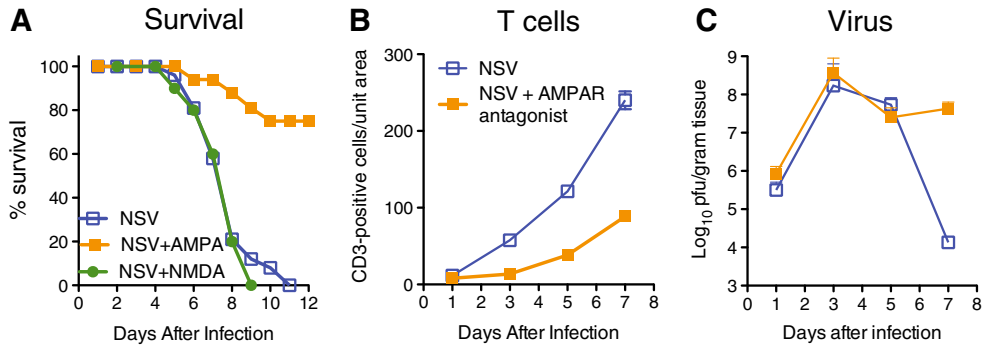

Fig. 2 Effects of treatment of neuroadapted Sindbis virus (NSV)-infected mice with glutamate receptor antagonists. **a** Survival of NSV-infected mice in the absence of treatment and in the presence of treatment for 7 days with the *N*-methyl-D-aspartate (NMDA) receptor antagonist MK-801 or the α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor antagonist GYKI-52466 [52]. Effects of treatment with AMPA receptor antagonist talampanel on T-cell infiltration into the brain **b** and on virus replication and clearance from the brain **c**

CNS (Fig. 2b) [57, 58]. Protection occurred despite a resultant delay in virus clearance (Fig. 2c).

Nonfatal SINV infection of hippocampal neurons leads to persistent behavioral abnormalities. At the peak of infectious virus titers in brain (day 5), mice are hyperactive, have decreased anxiety, and memory deficits that persist after clearance of infectious virus and resolution of clinical signs of disease. Mice treated with 6-diazo-5-oxo-l-norleucine, a glutamine antagonist that affects both the immune response by inhibiting lymphocyte proliferation and glutamate excitotoxicity by inhibiting neuronal glutaminase synthesis of glutamate had decreased inflammatory cell infiltration and cell death in the hippocampus [59]. Treatment inhibited development of clinical signs and memory deficits revealed by assessing contextual fear conditioning (Fig. 3), despite the presence of infectious virus and high levels of viral RNA [60].

Future Directions

For both AMPA receptor and glutamine antagonists, inhibition of the inflammatory response in the CNS prevented fatal disease, despite also slowing virus clearance, further indicating that neuronal damage and fatal disease are due to the immune response to virus-infected neurons rather than virus infection per se. However, when treatment is stopped, the immune response may be initiated along with neurologic disease. Therefore, although there are no approved drugs that inhibit alphavirus replication, ideal treatment would likely combine immune response inhibitors with an antiviral drug.

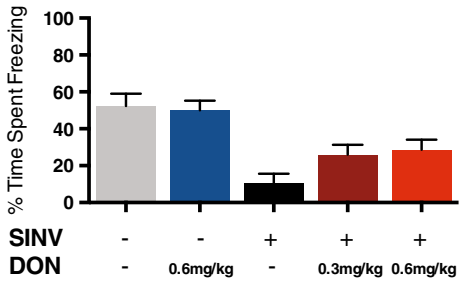

Fig. 3 Effect of treatment with 6-diazo-5-oxo-l-norleucine (DON) on hippocampal-dependent memory in Sindbis virus (SINV)-infected mice. Treatment with DON partially inhibited development of abnormalities in contextual fear conditioning 5 days after intranasal infection with SINV [55]

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