Available online at www.sciencedirect.com
ScienceDirect
Experimental Neurology 205 (2007) 461–470
Experimental Neurology
www.elsevier.com/locate/yexnr

The opioid receptor antagonist, naloxone, protects spinal motor neurons in a murine model of alphavirus encephalomyelitis

Natalie A. Prow [a], David N. Irani [b,*]

[a] Department of Molecular Microbiology and Immunology, The Johns Hopkins University Bloomberg School of Public Health, 615 N. Wolfe Street, Baltimore, MD 21205, USA
[b] Department of Neurology, The Johns Hopkins University School of Medicine, Baltimore, MD 21287, USA

Received 15 December 2006; revised 2 March 2007; accepted 5 March 2007
Available online 23 March 2007

Abstract

Spread of neuroadapted Sindbis virus (NSV) to motor neurons (MN) of the spinal cord (SC) causes severe hind limb weakness in C57BL/6 mice and models the paralysis that can accompany alphavirus and flavivirus encephalomyelitis in humans. The fate of spinal MN dictates the severity of NSV-induced paralysis, and recent data suggest that MN damage can occur indirectly via the actions of activated microglial cells. Because the opioid receptor antagonist, naloxone (NAL), blocks microglial-mediated neurodegeneration in other models, we examined its effects during NSV infection. Drug treatment prevented paralysis and enhanced the survival of MN without altering NSV tropism, replication, or clearance from SC tissue. Further studies showed that NAL most effectively inhibited paralysis in a 72-h window after NSV challenge, suggesting that the drug inhibits an early event in SC pathogenesis. Histochemical studies demonstrated that NAL blocked early microglial activation in SC tissue sections, and protein assays showed that the early induction of pathogenic IL-1β was blunted in SC homogenates. Finally, loss of glutamate transporter-1 (GLT-1) expression in SC, an astrocyte glutamate reuptake protein responsible for lowering toxic extracellular levels of glutamate and preventing MN damage, was reversed by NAL treatment. This GLT-1 loss proved to be highly IL-1β-dependent. Taken together, these data suggest that NAL is neuroprotective in the SC by inhibiting microglial activation that, in turn, maintains normal astrocyte glutamate homeostasis. We propose that drugs targeting such microglial responses may have therapeutic benefit in humans with related viral infections.

Keywords: Neuroprotection; Viral encephalitis; Motor neurons; Microglial activation; IL-1β; Glutamate transporter-1

Introduction

Alphaviruses and flaviviruses are important causes of fatal encephalitis in humans worldwide. Most of these infections are transmitted to mammalian hosts via the bite of infected mosquito vectors. One of these pathogens, West Nile virus (WNV), is newly arrived in the Western hemisphere where it has caused hundreds of fatal human encephalitis cases over the past few years (Granwehr et al., 2004). Neurological disease caused by this pathogen occurs with a wide array of clinical features that sometimes delays a specific diagnosis. Even when recognized promptly, however, antiviral therapies have proven ineffective and treatment is supportive. The focal occurrence of WNV epidemics thus far makes the development and widespread use of an effective vaccine an uncertain prospect for the near future. Therefore, unresolved issues related to WNV infection raise many public health concerns (Hayes et al., 2005).

Several related alphaviruses also cause epidemic, mosquito-borne encephalitis in humans, and a few of these viruses have been adapted for study in convenient animal models. One of these pathogens, Sindbis virus (SV), causes an acute encephalomyelitis in adult mice that closely reproduces many features of human alphavirus and flavivirus infections. A neuroadapted strain of SV (NSV) produces a lethal disease in susceptible hosts that evolves over a period of 7–10 days (Jackson et al., 1988). Following direct intracerebral inoculation, NSV spreads rapidly from the brain to the spinal cord (SC), where it causes a progressive lower motor neuron (MN) paralysis similar to WNV (Havert et al., 2000; Jackson et al., 1987; Kelley et al., 2003). Like other alphaviruses and flaviviruses, NSV selectively targets neurons with minimal infection of glial cells (Johnson et al., 1972). Neuronal fate

* Corresponding author. Fax: (410) 955 0105.
E-mail address: dirani@jhmi.edu (D.N. Irani).

0014-4886/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.expneurol.2007.03.013

determines outcome, with the neurovirulence of each SV strain directly related to the extent of neuronal destruction it produces (Lewis et al., 1996). As an added layer of complexity, different neuronal populations undergo different types of cell death following NSV infection (Havert et al., 2000; Lewis et al., 1996), and many non-infected neurons are also damaged via bystander mechanisms, believed to be glutamate-mediated excitotoxicity (Nargi-Aizenman and Griffin, 2001). This bystander injury has led to the suggestion that host immune responses somehow contribute to NSV pathogenesis (Liang et al., 1999; Kimura and Griffin, 2000, 2003), although the degree to which neuronal damage occurs via host-derived rather than direct viral-induced mechanisms remains poorly understood.

Microglia are the principal endogenous immune cells of the central nervous system (CNS) (Lawson et al., 1990), and these cells become activated in response to injury, infection, or inflammation (Gehrmann et al., 1995; Perry and Gordon, 1991). Upon activation, microglia proliferate, change morphology, and assume many macrophage-like functions. When neurons die, microglia contribute to the removal of cellular debris (Streit, 1996; Streit et al., 1988). Activated microglia may also actively contribute to neuronal cell death since therapies that inhibit microglial activation result in increased neuronal survival in numerous experimental models (Rogove and Tsirka, 1998; Thanos et al., 1993; Yrjanheikki et al., 1998). Activated microglia release cytotoxic substances such as IL-1β, TNFα, superoxide anion, and nitric oxide, all of which may contribute to neuronal cell death *in vitro* and *in vivo* (Bronstein et al., 1995; Liu et al., 2000a,b; Takeuchi et al., 1998). Little, however, is known about the role of activated microglia in the pathogenesis of alphavirus and flavivirus encephalomyelitis.

The classic opioid receptor antagonist, naloxone (NAL), has been shown to block the production of inflammatory mediators by activated microglial cells *in vitro* (Liu et al., 2000a, b; Chang et al., 2000; Liu et al., 2002), and to attenuate neurological deficits in animal models of stroke, SC injury, and Parkinson's disease *in vivo* (Faden et al., 1981; Hosobuchi et al., 1982; Liao et al., 2003; Lu et al., 2000). Indeed, an inflammatory basis for at least some of the neuronal injury that occurs in these diverse disorders has long been hypothesized to derive from microglial responses that consistently accompany them. Products of activated microglia are also implicated in the pathogenesis of various CNS viral infections, particularly those caused by HIV and HSV (Glass and Wesselingh, 2001; Marques et al., 2006; Wesselingh and Thompson, 2001). As previous studies from our group have shown evidence of widespread microglial activation in the CNS of SV-infected mice (Tyor et al., 1990), we investigated the effects of NAL in the more virulent NSV encephalomyelitis model. Here, we report that the drug prevents paralysis and limits MN destruction in a measurable therapeutic window, and that its mechanism of action appears to involve the blockade of local microglial activation and IL-1β production in the SC. This leads to normalized glutamate transporter expression and suggests that glutamate-mediated excitotoxicity is being prevented in NSV-induced encephalomyelitis. Taken together, these studies demonstrate that neuroprotective agents targeting such detrimental host immune responses have therapeutic potential in these otherwise untreatable infections.

Materials and methods

Animal manipulations

All animal procedures had received prior approval from the Johns Hopkins institutional animal care and use committee and were performed under isoflurane anesthesia (Abbott Laboratories, North Chicago, IL). Five- to 6-week-old C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Age-matched B6.129F1 (IL-1β$^{+/+}$) and IL-1β-deficient (IL-1β$^{-/-}$) mice were purchased from Taconic (Hudson, NY). To induce encephalomyelitis, all animals received intracerebral inoculations of 1000 plaque-forming units (PFU) of NSV in a volume of 20 μl of PBS into the right cerebral hemisphere. Mice were monitored daily for signs of disease in accordance with approved animal protocols. Twenty animals per group were used for all experiments unless otherwise noted. For those experiments where tissue samples were not being collected for ex vivo analysis, each animal was scored daily into one of the following categories: (0) normal or minimally affected, (1) mild paralysis (some weakness of one or both hind limbs), (2) moderate paralysis (weakness of one hind limb, paralysis of the other hind limb), (3) severe paralysis (complete paralysis of both hind limbs), or (4) dead. Many animals were treated with NAL at 20, 50, or 100 mg/kg (naloxone hydrochloride (−) isomer, Sigma Chemical Company, St. Louis, MO, catalogue number N7758) or saline given as a daily intraperitoneal injection starting at different times after viral challenge. The significance of clinical differences between groups was calculated by Kaplan–Meier analysis where noted (GraphPad Prism version 4.0, GraphPad Software Inc., San Diego, CA).

Tissue viral titrations

To measure the amount of infectious virus present in lumbar SC tissues, animals were perfused with PBS and cords were extracted, weighed, snap frozen on dry ice, and stored at −80 °C until viral titration assays were performed. At the time of these titrations, 10% (w/v) homogenates of each sample were prepared in MEM supplemented with 2% fetal bovine serum, and serial 10-fold dilutions of each homogenate were assayed for plaque formation on monolayers of BHK-21 cells, according to standard protocols used in our laboratory (Kerr et al., 2002). The results presented are the mean ± standard error of the mean (SEM) of the $\log_{10}$ of viral plaque-forming units per gram of tissue derived from 3 animals at each time point.

Histology

Animals designated for histological analyses were sequentially perfused with chilled PBS and 4% paraformaldehyde in PBS via a transcardial approach. All tissues were then post-fixed in 4% paraformaldehyde overnight at 4 °C, after which they were embedded in paraffin for sectioning. All lumbar