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Glutamine antagonist-mediated immune suppression decreases pathology but delays virus clearance in mice during nonfatal alphavirus encephalomyelitis

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ABSTRACT

Infection of weanling C57BL/6 mice with the TE strain of Sindbis virus (SINV) causes nonfatal encephalomyelitis associated with hippocampal-based memory impairment that is partially prevented by treatment with 6-diazo-5-oxo-l-norleucine (DON), a glutamine antagonist (Potter et al., J Neurovirol 21:159, 2015). To determine the mechanism(s) of protection, lymph node and central nervous system (CNS) tissues from SINV-infected mice treated daily for 1 week with low (0.3 mg/kg) or high (0.6 mg/kg) dose DON were examined. DON treatment suppressed lymphocyte proliferation in cervical lymph nodes resulting in reduced CNS immune cell infiltration, inflammation, and cell death compared to untreated SINV-infected mice. Production of SINV-specific antibody and interferon-gamma were also impaired by DON treatment with a delay in virus clearance. Cessation of treatment allowed activation of the antiviral immune response and viral clearance, but revived CNS pathology, demonstrating the ability of the immune response to mediate both CNS damage and virus clearance.

1. Introduction

Recent epidemics of rash, arthritis and encephalomyelitis emphasize the importance of arthropod-borne viruses, primarily alphaviruses and flaviviruses, to threaten public health (Lubelczyk et al., 2013; ; Molaei et al., 2015; Weaver et al., 1996). Encephalomyelitis induced by infection with alphaviruses can be severe, with outcome dependent on the virus species (Babi et al., 2014; Baig et al., 2014; Bruyn and Lennette, 1953; Ethier and Rogg, 2012; Harvala et al., 2009; Lury and Castillo, 2004; Mancao et al., 2009; Przelomski et al., 1988; Reddy et al., 2008; Rivas et al., 1997; Rozdilsky et al., 1968; Schultz et al., 1977; Vilcarromero et al., 2010). Eastern equine encephalitis virus is most neurovirulent with fatalities most commonly in infants and children and frequent neurologic sequelae in those that survive (Deresiewicz et al., 1997; Finley et al., 1955). Currently, no treatments beyond supportive care are available, and no licensed vaccines for alphavirus encephalomyelitis are approved for non-military human use, so there is a need to identify new approaches to treatment (Go et al., 2014).

Infection of mice with Sindbis virus (SINV) leads to encephalomyelitis, providing a model for evaluating clinical disease and central nervous system (CNS) pathology induced by alphavirus infection. SINV preferentially infects neurons in mice, particularly the hippocampal neurons of the brain and the motor neurons of the spinal cord, and outcome is dependent on the strain of infecting virus and genetic background of the mouse (Kimura and Griffin, 2003; Griffin, 2011). Weanling C57BL/6 mice infected with the neurovirulent NSV strain of SINV develop fatal encephalomyelitis while mice infected with the less virulent TE strain recover with persistent hippocampal-dependent memory deficits as measured by fear conditioning (Jackson et al., 1988; Potter et al., 2015).

The immune response to alphavirus infection of the CNS presents a

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double-edged sword: while the immune response is necessary for bringing virus replication and production under control, it is also responsible for many of the pathological changes and neurological damage produced. After infection with the neurovirulent NSV strain of SINV, fatal encephalomyelitis coincides with infiltration of $CD4^+$ and $CD8^+$ T cells into the brain (Kulcsar et al., 2014), and in studies of mice lacking components of the immune response, particularly T cell deficits, mortality and severity of disease are decreased (Rowell and Griffin, 2002; Kimura and Griffin, 2003). Furthermore, previous studies have shown that inhibition of the immune response and glutamate excitotoxicity with 2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl) propranoic acid (AMPA) receptor antagonists can protect NSV-infected mice from fatal encephalomyelitis (Greene et al., 2008; Nargi-Aizenman et al., 2004).

DON is a diazo-containing glutamine analog that acts as a broad irreversible competitive inhibitor of several glutamine-utilizing enzymes (Thangavelu et al., 2014). During growth and proliferation, T cells preferentially use glutamine instead of glucose as an energy source, and rapidly dividing activated T cells show increased glutamine uptake and metabolism (Carr et al., 2010; Maciolek et al., 2014). Therefore, glutamine antagonism can inhibit lymphocyte proliferation (Wang et al., 2011). In the CNS, the excitatory neurotransmitter glutamate is synthesized from glutamine by the amidohydrolase enzyme glutaminase. Excess production of glutamate leads to neuronal death through glutamate excitotoxicity, so inhibition of glutaminase via DON might abrogate this process (Sattler and Tymianski, 2001). In support of this approach, mice infected with the fatal NSV strain of SINV show reduced mortality with DON treatment (Manivannan et al., 2016), and in the less virulent TE strain of SINV, DON reduces the development of clinical disease and hippocampus-dependent memory deficits (Potter et al., 2015). To determine the mechanism(s) by which DON improves outcome from nonfatal alphavirus encephalomyelitis, we have examined the effect of treatment on both pathological changes and virus clearance in the CNS.

2. Materials and methods

2.1. Sindbis virus infection and DON administration

The TE strain of SINV (Lustig et al., 1988) was grown and assayed by plaque formation in baby hamster kidney (BHK) cells. Four to six-week-old male C57BL/6 mice (Jackson Laboratory) were infected intranasally with 10^5 pfu SINV in 20 μL PBS or mock-infected with 20 μL PBS while under light isoflurane anesthesia. DON was intraperitoneally administered once a day from the day of infection to 7 days post infection (DPI) at a dose of 0.3 mg/kg (low dose) or 0.6 mg/kg (high dose) as previously described (Potter et al., 2015). Control animals received the same volume of PBS. All studies were done in accordance with protocols approved by the Johns Hopkins University Institutional Animal Care and Use Committee.

2.2. Tissue collection

At two time points during drug administration (5 and 7 DPI) and at two time points following cessation of treatment (9 and 11 DPI), mice were euthanized by isoflurane overdose and perfused with 15 mL ice cold PBS following blood collection by cardiocentesis. Blood was allowed to coagulate in BD Microtainer serum separator tubes and centrifuged for 15 min at 1500 RCF. Serum was collected and stored at −20 °C. For RNA and plaque assay analysis, brains and spinal cords were collected, flash-frozen and stored at −80 °C. For histopathology and immunohistochemistry, mice were perfused with 40 mL cold 4% paraformaldehyde (PFA), and brains and spinal columns were collected. Brains were divided into three coronal sections using an Adult Mouse Brain Slicer (Zivic Instruments), fixed overnight in 4% PFA at 4 °C, and washed in ice-cold PBS. Spinal columns were trimmed of excess soft tissue, fixed overnight in 4% PFA at 4 °C, and then decalcified on a rotator for 24–36 h in a 10% sodium citrate/22% formic acid solution. Spinal columns were washed in ice-cold PBS and the L4-L6 regions were isolated. Tissues were embedded in paraffin for sectioning and staining.

2.3. Mononuclear cell isolation

Single cell suspensions were made from cervical lymph nodes (CLNs), brains, and spinal cords pooled from two to five mice per group per time point as previously described (Baxter and Griffin, 2016). Briefly, CLNs were dissociated using a gentleMACS Dissociator (Miltenyl Biotec), and red blood cells were lysed using ammonium chloride (Sigma-Aldrich). Brains and spinal cords were dissociated in RPMI containing collagenase and DNase in C tubes using a gentleMACS Dissociator. Mononuclear cells were separated from red blood cells and myelin debris on a 30%/70% Percoll gradient (GE Healthcare) for 30 min at 4 °C. Live mononuclear cells from all tissues were quantified by trypan blue exclusion.

2.4. Flow cytometry

Cells were stained and analyzed by flow cytometry as previously described (Baxter and Griffin, 2016). Briefly, 10^6 live cells were stained with violet LIVE/DEAD Fixable Dead Cell Stain (Life Technologies) for 30 min, anti-mouse CD16/CD32 (BD Pharmingen) for 15 min, and monoclonal antibodies against CD45 (clone 30-F11), CD3 (clone 17A2), CD4 (clone RM4-5), CD8a (clone 53-6.7), and CD19 (clone 1D3) from Ebioscience or BD Pharmingen for 30 min. Cells were run on a BD FACSCanto II cytometer using BD FACSDiva software, version 8, and analyses were carried out using FlowJo software, version 8. Cells were characterized as follows: CD4 T cells ($CD45^{hi}CD3^+CD4^+$), CD8 T cells ($CD45^{hi}CD3^+CD8^+$), and B cells ($CD45^{hi}CD3^-CD19^+$). For CLN and brain, total mononuclear cell counts were from three independent experiments, and for spinal cord, as well as $CD4^+$, $CD8^+$ and $CD19^+$ cells for all tissues, counts were from two independent experiments. At 11 DPI, the SINV, 0.6 mg/kg DON group had data from one fewer experiment than the others (one to two independent experiments).

2.5. Histology and immunohistochemistry

Three to four 10 μm brain sections per mouse were stained with H & E, coded, and scored as previously described (Rowell and Griffin, 1999) using a 0–3 scale. A score of 0 was given when there was no detectable inflammation, a score of 1 for brains with one to two small inflammatory foci per section, a score of 2 for moderate inflammatory foci in up to 50% of 10X fields, and a score of 3 for moderate to large inflammatory foci in greater than 50% of 10X fields. An additional point was added for excessive parenchymal cellularity, allowing for a maximal score of 4.

Three to four 10 μm lumbar spinal cord sections per mouse were stained with H & E, coded, and scored using a 0–2 scale adapted from the brain scoring system. A score of 0 was given for no detectable inflammation, a score of 1 for one to two small inflammatory foci per section, and a score of 2 for greater than two inflammatory foci per spinal cord or for spinal cords with moderate to marked inflammatory foci. An additional point was added for excessive parenchymal cellularity, allowing for a maximal score of 3.

For immunohistochemical staining, 10 μm brain sections were deparaffinized and hydrated. Antigen retrieval was performed by boiling slides in 0.01 M sodium citrate, pH 6.0 (glial fibrillary acidic protein, GFAP), and endogenous peroxidase was quenched with 3% H_2O_2 in methanol for 10 min (ionized calcium binding adaptor molecule 1, IBA1 and glutamate transporter 1, GLT1). Slides were blocked in 10% normal goat serum (NGS), and incubated with primary antibody diluted in PBS +5% NGS +0.04% TritonX (GFAP 1:1000,