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Role of chemokines in the enhancement of BBB permeability and inflammatory infiltration after rabies virus infection

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ABSTRACT

Induction of innate immunity, particularly through the induction of interferon and chemokines, by rabies virus (RABV) infection has been reported to be inversely correlated with pathogenicity. To further investigate the association between the expression of chemokines and RABV infection, laboratory-attenuated RABV (B2C) and wild-type (wt) RABV (DRV) were administered to Balb/c mice intramuscularly. Chemokine expression, inflammatory cell infiltration, and blood–brain barrier (BBB) permeability were evaluated at various time points after infection. At day 3 post-infection (p.i.) there was very little inflammation in the central nervous system (CNS) and BBB permeability did not change in mice infected with either virus when compared with mock-infected mice. At 6 day p.i., infection with B2C induced the expression of inflammatory chemokines and infiltration of inflammatory cells into the CNS, while these changes were minimal in DRV-infected mice. Furthermore, infection with B2C significantly enhanced BBB permeability comparing to infection with DRV. Among the upregulated chemokines, the expression of IP-10 was best correlated with infiltration of inflammatory cells into the CNS and enhancement of BBB permeability. These data indicate that laboratory-attenuated RABV induces expression of chemokines and infiltration of inflammatory cells into the CNS. Upregulation of chemokines by B2C may have triggered the change in BBB permeability, which helps infiltration of inflammatory cells into the CNS, and thus attenuation of RABV.

1. Introduction

Rabies is one of the most important zoonotic infections and still causes more than 55,000 human deaths each year (Martinez, 2000). Most of the human rabies cases occur in Asia and Africa where dog rabies is prevalent (Anonymous, 1992; Fu, 1997). In the United States, dog rabies has almost been eliminated through massive vaccination during the past 6 decades (Blanton et al., 2006). However, bat (particularly the silver-haired bat) rabies has emerged to be responsible for most of the human rabies cases in the past 20 years (CDC, 2003; Morimoto et al., 1996; Rupprecht et al., 1997). Once clinical signs develop, rabies is always fatal (Anonymous, 1992; Fu, 1997). Despite the lethality of rabies, only mild inflammation and little neuronal destruction were observed in the central nervous system (CNS) of rabies patients (Miyamoto and Matsumoto, 1967; Murphy, 1977). Alternatively, laboratory-attenuated RABV induces extensive inflammation and neuronal degeneration in experimentally infected animals (Miyamoto and Matsumoto, 1967; Murphy, 1977). To understand the differential effects between wild-type (wt) and attenuated viruses, mice were infected with two RABV strains, one laboratory-attenuated RABV and the other wt RABV, and compared the host responses to infection (Wang et al., 2005). It was found that laboratory-attenuated RABV induced extensive inflammation, apoptosis, and neuronal degeneration in the CNS; however, wt RABV caused little or no neuronal damage. Furthermore, laboratory-attenuated RABV induced the expression of genes associated with innate immune responses, particularly type 1 interferon (IFN-α and -β), chemokines and complements while many of these genes were not activated in mice infected with wt RABV (Wang et al., 2005). The induction of innate immunity has been confirmed by others using laboratory-attenuated viruses to infect mice or neuronal cells (Johnson et al., 2006; Nakamichi et al., 2004; Prehaud et al., 2005). Induced innate immune response genes include inflammatory chemokines (including RANTES, MIP-1α, IP-10) and cytokines (IL-6, IL-1β, and TNF-α), IFN and IFN-related genes (IFN-α/β, STAT1), as well as Toll-like receptors (TLRs) (Johnson et al., 2006; Nakamichi et al., 2004; Prehaud et al., 2005). These observations led to the hypothesis that laboratory-attenuated RABV is a potent inducer of host innate immunity (Wang et al., 2005). Innate immune response, especially

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proinflammatory cytokines and chemokines can recruit immune cells to the site of infection to remove pathogens, which might be an important mechanism of RABV attenuation.

Recently, it has been reported that wt and laboratory-attenuated RABVs differentially induce changes in the blood–brain barrier (BBB) permeability (Fabis et al., 2008; Phares et al., 2007; Roy and Hooper, 2007; Roy et al., 2007). The BBB was more permeable in mice infected with laboratory-attenuated CVS-F3 than mice infected with silver-haired bat rabies virus (SHBRV). The former is an antibody escape mutant derived from CVS virus (Dietzschold et al., 1983) and the latter is a wt RABV isolated from a human patient (Morimoto et al., 1996). It was reasoned that enhancement of BBB permeability allows immune effectors to cross the BBB and enter the CNS. Indeed, adoptive transfer of immune cells isolated from mice infected with laboratory-attenuated RABV which resulted in clearance of wt RABV from the CNS (Roy et al., 2007). These studies suggest that changes in BBB permeability are associated with clearance of the apathogenic RABV from the CNS (Phares et al., 2007; Roy et al., 2007). Alternatively, failure to increase the permeability of BBB leads to disease in wt RABV-infected mice (Roy et al., 2007).

The present study attempts to determine the contributions of the innate immune response, especially the role of inflammatory chemokines, in the enhancement of BBB permeability and the outcome of RABV infection. If increased BBB permeability is the major contributor towards survival, it is important to determine the mechanism that is responsible for this change in RABV-infected animals. Therefore, expression of chemokines, infiltration of inflammatory cells, and enhancement of BBB permeability was investigated in mice infected with laboratory-adapted or wt RABV. It was found that expression of chemokines was closely associated with infiltration of inflammatory cells and increases in BBB permeability. Among the chemokines investigated, the expression of IP-10 was best correlated with such changes in mice infected with laboratory-attenuated RABV. The data indicate that laboratory-attenuated RABV upregulates the expression of chemokines, which increases infiltration of inflammatory cells into the CNS, triggering changes in the BBB permeability. This in turn helps more infiltration of effector cells into the CNS resulting in attenuation of RABV virulence.

2. Materials and methods

2.1. Viruses, antibodies

Four RABVs were used in this study and they are SHBRV, DRV, B2C, and SN-10. SHBRV is a wt RABV isolated from a human patient (Morimoto et al., 1996). DRV is a wt virus isolated from a dog (Dietzschold et al., 2000). B2C is a laboratory-attenuated virus isolated from challenge virus standard (CVS-24) by serial passaging in BHK cells (Morimoto et al., 1998). SN-10 is a cloned virus derived from the attenuated SAD B19 vaccine strain (Schnell et al., 1994). Virus stocks were prepared as described (Sarmento et al., 2005; Wang et al., 2005). Briefly, one-day-old suckling mice were inoculated with 10 μl of viral inoculum by the intracerebral (IC) route. When moribund, mice were euthanized and brains were removed. A 10% (w/v) suspension was prepared by homogenizing the brain in DMEM. The homogenate was centrifuged to remove debris and the supernatant collected and stored at −80 °C. Fluorescein isothiocyanate (FITC)-conjugated antibody against the RABV nucleoprotein (N) protein was purchased from FujiRebio (FujiRebio Diagnostic Inc., PA). Anti-RABV N monoclonal antibody 802-2 (Hamir et al., 1995) was obtained from Dr. Charles Rupprecht, Center for Disease Control and Prevention (CDC). Anti-RABV glycoprotein (G) polyclonal antibody was prepared in rabbits as described (Fu et al., 1993) and has been shown to have similar affinity to the G from wt SHBRV and laboratory-adapted N2C (Yan et al., 2001). Anti-CD3 polyclonal antibody was purchased from Dako (Dako North America, CA).

2.2. Mice

Female ICR mice at the age of 4–6 weeks were purchased from Harlan (Harlan, IN) and Balb/c mice at 6–8 weeks of age were purchased from NCI (NCI-Frederick, MD). Mice were housed in temperature- and light-controlled quarters in the Animal Facility, College of Veterinary Medicine, University of Georgia. All animal experiments were carried out as approved by the Institutional Animal Care and Use Committee.

2.3. Real-time quantitative RT-PCR

Brains and spinal cords were removed from infected mice at indicated time points and flash frozen on dry ice before being stored at −80 °C. RNA was extracted from these tissues using Trizol following the manufacturer's instructions and used for real-time PCR as described previously (Wang et al., 2005). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous reference gene. Amplification primers of the proinflammatory genes are listed in a previous paper (Wang et al., 2005). To determine virus replication and RNA transcription in each sample, virus specific N mRNA and genomic RNA were measured by quantitative RT-PCR. cDNA standards were made from RABV N gene. Standard curves were constructed with C_t values obtained using dilutions of the synthetic standards. The mRNA and genomic RNA copy numbers in each sample were normalized to the respective copy number derived from the standard curve.

2.4. BBB integrity

BBB permeability was assessed using a modification of a previously described technique in which Na-fluorescein (NaF) is utilized as a tracer molecule (Phares et al., 2007). Mice received 100 μl of 10% NaF in PBS intravenously under anesthesia. After 10 min to allow circulation of the NaF, cardiac blood was collected and the animals were transcardially perfused with PBS. Spinal cord or brain tissues were homogenized in cold 7.5% trichloroacetic acid (TCA) and centrifuged for 10 min at 10,000 × *g* to remove insoluble precipitates. After the addition of 0.25 ml 5N NaOH, the fluorescence of a 100 μl supernatant sample was determined using a BioTek Spectrophotometers (Bio-Tek Instruments, Inc.) with excitation at 485 nm and emission at 530 nm. Standards (125–4000 μg/ml) were used to calculate the NaF content of the samples. NaF uptake into tissue is expressed as (μg fluorescence spinal cord/mg tissue)/(μg fluorescence sera/ml blood) to normalize values for blood levels of the dye at the time of tissue collection (Phares et al., 2007).

2.5. Histopathology and immunohistochemistry

For histopathology and immunohistochemistry, animals were anesthetized with ketamine–xylazine at a dose of 0.1 ml/10 g body weight and then perfused by intracardiac injection of PBS followed by 10% neutral buffered formalin as described previously (Li et al., 2005). Brains, spinal cords and dorsal root ganglia (DRG) were removed and paraffin embedded for coronal sections (4 μm). To de-paraffin, slides were heated at 60 °C for 25 min and then dipped in CitriSolv (Fisher Scientific, PA) three times for 5 min and dried until chalky white. After de-paraffinization, slides were stained with hematoxylin and eosin (H&E). Slides were heated in antigen unmasking solution (Vector Laboratories, CA) above 90 °C for 20 min and naturally cooled down to room temperature. Anti-RABV N monoclonal antibody 802-2 was used to detect the viral antigen. The primary antibody and then secondary antibody (biotinylated)