ROLE OF CHEMOKINES IN THE ENHANCEMENT OF BBB PERMEABILITY AND INFILTRATION OF INFLAMMATORY CELLS AFTER RABIES VIRUS INFECTION

by

Yi Kuang

(Under the Direction of Zhen Fu)

ABSTRACT

Rabies is one of the most important zootonic diseases. Once clinical signs develop, rabies is almost always fatal. More than 55,000 people die of rabies and millions more receive rabies postexposure prophylaxis each year all over the world. Despite the lethality of rabies, only mild inflammation and little neuronal destruction are observed in the central nervous system (CNS) of rabies patients. On the other hand, laboratory-attenuated rabies virus (RV) induces extensive inflammation and neuronal degeneration in experimentally infected animals. Previous studies have been conducted to investigate what causes the differential effects. It was found that laboratory-attenuated RV induces strong innate immune responses including the expression of inflammatory chemokines and cytokines, IFN and IFN-related genes, as well as Toll-like receptors (TLRs). These observations led

to the hypothesis that laboratory-attenuated RV is a potent inducer of host innate immunity, which might be an important mechanism of RV attenuation.

The present study was conducted to further investigate the association between the expression of chemokines and RV infection. Laboratory-attenuated RV (B2C) and wild-type (wt) RV (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 6 day post infection (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 compared 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 RV induces chemokines expression 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 the effects of RV infection.

INDEX WORDS: rabies, innate immunity, chemokine, IP-10, MIP-1a, Rantes

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by

YI KUANG

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YI KUANG

Major Professor: Zhen Fu

Committee: Elizabeth Howerth
Elizabeth Uhl

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
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DEDICATION

I dedicate this work to my parents and family members for all their unconditional support, your support and love make this work possible.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Rabies disease:

Rabies is one of the oldest diseases and dates back to 23rd century B.C. The first written record of rabies was documented in the Eshnunna Code. Aristotle (4th century BC) recorded that rabies was caused by animal bites. The word *lyssa* was first used by Plato (4th century BC) to describe erotic passion. Latin word *rabere*, which means raging, furious, savage, or madness (Baer, 2007). Caelius Aurelians (in first century AD) described patient suffering, thirst and fear of water associated with the disease. Galen (2nd century AD) believed that only dogs were susceptible to rabies. During the Middle Ages, rabies in dogs was reported in many countries in the old world(Baer, 2007). In 1709, rabies was reported in Mexico, North America for the first time (Baer, 2007). The era of animal experiment of rabies began in 1804 when George Gottfried Zinke first demonstrated that rabies was able to be transmitted from saliva of rabid dogs (Baer, 2007). In 1879, Victor Galtier showed that the transmission of rabies to rabbits by injection or bite (Baer, 2007). Pasteur was the first to use the desiccated tissue from rabies-infected rabbits for vaccination. On July 6th 1885, a 9-year old boy, Joseph Meister, who was repeatedly bitten by a rabid dog, survived after receiving postexposure treatment with the Pasteur vaccine. This was the first time in the history there was

treatment for the disease. From that time on, Pasteur vaccine and modifications were used all over the world to control rabies (Baer, 2007).

Rabies is one of the most important zoonotic infections and causes more than 55,000 human death each year (Martinez, 2000). More than 90% of all human deaths from rabies occur in Africa and Asia (Bingham, 2005). In some countries, such as India, China, and the Philippines, rabies accounts for thousands of human deaths every year. In China, 103,200 people died of rabies in four epidemic waves between 1950 and 2004 (Webpage, 2006). Most of these human rabies cases were transmitted by dogs. In North and South America as well as Europe, dog rabies has been brought under control through animal vaccination and as a consequence, the number of human cases has been reduced considerably (Krebs et al., 1996). However, wildlife rabies has become a major concern. Approximately 92% of the reported animal rabies cases were found in wildlife, and only 8% in domestic animals (Blanton et al., 2007). During 2006, 49 states and Puerto Rico reported 6,940 cases of rabies in animals and 3 cases in humans to the CDC, representing an 8.2% increase from the 6,417 cases in animals and 1 case in a human reported in 2005. As a consequence, an estimated 40,000 people receive rabies postexposure prophylaxis each year (Gibbons, 2002).

Rabies virus structure

The rabies virus (RV) is enveloped and bullet-shaped virus belonging to the genus *Lyssavirus*, family Rhabdoviridae (Wunner, 2007). Its genome is non-segmented and negative-sense RNA. RV virons are approximately 180 nm long and 75 nm wide. RV genome encodes five proteins: nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and polymerase (L) (Wunner, 2007). The N, P, and L protein form the

ribonucleoprotein (RNP) complex with viral RNA. G and M proteins are associated with the lipid envelop which surrounds the RNP core. The N protein plays important roles in regulating RNA transcription and replication. The G protein is the only surface protein that is capable of stimulating the production of virus neutralizing antibodies. RV G also plays a crucial role in the process of RV pathogenesis (Dietzschold B, 1996b) binding to specific neural receptors for entering the nervous system (Cox et al., 1977). The RV G protein determines the distribution pattern of a particular virus strain in the brain. It is believed that the G protein mediates the fusion of the viral envelope with endosomal membranes to release the infectious core into the cytosol for virus transcription and replication (Dietzschold et al., 1983; Morimoto et al., 1999).

Rabies Virus Replication

The replication cycle of RV is typical of most non-segmented negative-strand RNA viruses. The early events include attachment, penetration, and uncoating, which allows the viral nucleocapsid to be released into the host cell cytoplasm. The middle phase is composed of viral genome transcription, translation and replication. The late phase includes budding, which released the virus for subsequent infections (Albertini et al., 2008; Dietzschold B, 1996b; Finke and Conzelmann, 2005).

Attachment, penetration, and uncoating: RV infection is initiated by G protein which binds to specific receptors on the target host cell surface (Broughan and Wunner, 1995; Tuffereau et al., 2001). Several different neuronal surface molecules have been identified as potential receptors, including the nicotinic acetyl choline receptor (AchR), the neural cell adhesion molecule (NCAM), and the low-affinity nerve-growth factor receptor p75NTR (Wunner, 2007). Once bound, the virus is uptaken by endocytosis and the virus

envelop fuses with endosomal membrane, releasing the viral cores into the cytoplasm, allowing infection to proceed (Wunner, 2007).

Transcription and replication: Transcription occurs in the cytoplasm of infected host cells. The negative-strand RNA genome is transcribed into mRNA by the RNA-directed RNA polymerase (RdRP). Transcription begins at the 3' end of the genome where the polymerase produces a 55-nucleotide leader RNA. Different froming the RNA transcript, the leader is neither capped nor polyadenylated. The individual mRNAs encoding the N, P, M, G and L proteins are synthesized sequentially (Wagner and Rose, 1996). The transcriptional order follows the gene order. Then, the individual mRNAs are capped and polyadenylated. Following viral proteins synthesis, viral transcription is switched to replication (Wagner and Rose, 1996). Rabies virus replication requires active, ongoing translation, particularly of viral N and P proteins (Dietzschold B, 1996a). Once N, P and L proteins are synthesized, these proteins and newly replicated genomic RNA are associated in the cytoplasm and forming the RNP core. Then the RNP core associates with M protein forming a coiled structure called the skeleton. The M protein condenses the RNP core in the cytoplasm and directs the RNP core to the membrane in preparation for budding (Wagner and Rose, 1996). The G protein interacts with internal virion components, most likely the M or N proteins, and subsequent envelopment of the particles leads to the last phase of the infection cycle (Wagner and Rose, 1996; Wunner, 2007).

Rabies pathogenesis

The virus may enter the peripheral nervous system directly, or may replicate in muscle tissue after entering the host, remaining at or near the site of introduction for most of the

incubation period (Jackson, 2007). The incubation time may vary. The infection of muscle fibers may be a critical pathogenetic step for the virus to gain access to the peripheral nervous system (Jackson, 2002).

Early events of infection: The virus may enter the peripheral nervous system via the neuromuscular junctions, and moves rapidly centripetally to the central nervous system, particularly to the nearest sensory or motor neuron in the dorsal root ganglion or anterior horn of the spinal cord where it replicates. Nicotinic acetylcholine receptor (nAChR), neuronal cell adhesion molecule (NCAM), and p75 neurotrophin receptor (p75NTR) are the three receptors that mediate rabies virus entering into nerve endings (Fu, 1997; Jackson, 2007).

Spread to and within the CNS: Rabies virus spreads from the peripheral to the CNS by retrograde transport. The speed is usually between 50 and 100mm/day. Rabies virus glycoprotein may play an important part in virus transportation. Once CNS neurons become infected, there is rapid dissemination of rabies virus along neuroanatomical pathways (Jackson, 2007). Rabies virus also spreads within the CNS, as in the peripheral nervous system, by fast axonal transport. The rabies glycoprotein is important and necessary for trans-synaptic spread of rabies virus from one neuron to another. Once the virus reaches the brain, it rapidly causes encephalitis and symptoms in appear (Jackson, 2007).

Late stage of infection: Rabies virus can be transported centrifugally to many peripheral tissues and organs, such as respiratory tract, cornea, skin of the head and neck, adipose tissue, adrenal medulla, and renal parenchyma. Extremely high viral titers are directly reached by way of efferent secretory nerves to a primary exit portal, the acinar cells of

the salivary glands, often exquisitely timed to host aberrant behaviors which enhances RV shedding potential and its natural perpetuation (Jackson, 2007). Rabid patients develop severe agitation, depression, hydrophobia, and paralysis followed by impaired consciousness and coma. Patients eventually die of circulatory insufficiency, cardiac arrest, and respiratory failure (Jackson, 2007).

Gross examination of the brain shows mild congestion of the meningeal vessels and histopathologic findings are usually mild. This suggests that the neurologic disease in rabies must result from neuronal dysfunction rather than morphologic changes (Rossiter and Jackson, 2007). It is believed that the combination of rabies virus infection of the neuron system and host immunity cause neuronal dysfunction (Wagner and Rose, 1996). A variety of studies of rabies virus infection in experimental animals and in vitro experiments have provided evidence that RV cause defective in neurotransmission, acetycholine, serotonin, or γ-amino-*n*-butyric acid (GABA) (Jackson, 2007; Ladogana et al., 1994). The neuronal dysfunction perhaps could be explained by a defect in cholinergic synaptic neurotransmission. Rabies virus may also impair ion channels and electrophysiologic properties of neurons. Dysfunction of ion channels has been demonstrated in infected culture cells and the induction of inducible nitric oxide synthase mRNA and the increase in the levels of nitric oxide have been shown in rabies virus-infected rodents (Iwata et al., 1999). Rabies viruses may induce apoptosis of T cells that migrate into the nervous system, which would limit inflammation and guaranty spread of the virus (Thoulouze et al., 1997). The precise mechanism of neuron dysfunction and pathogenesis in RV infection is still poorly understood. Abnormal behavior in rabies infected individuals is associated with lesions in multiple locations in the brain. Early and

selective brain stem infection in rabies would allow centrifugal spread of the virus to salivary glands as well as involvement of the serotonergic system in the raphe nuclei, resulting aggressive behavior of animals with adequate cognitive and motor function in order to execute successful viral transmission by biting (Jackson, 2007; Jackson and Rossiter, 1997).

Immune response against rabies virus

It is unlikely that rabies virus triggers a primary adaptive immune response in the nervous system (Lafon, 2007). Nevertheless, once in the nervous system, there is evidence that RV triggers an early innate immune response, characterized by antiviral, chemoattractive and inflammatory response in which infected neurons play an active part. Inflammatory cytokines IFNα/β, IL-1, IL-6, TNF-α, as well as chemokines IP-10, RANTES, MIP-1α, can be detected in animal models (Jackson et al., 2006; Johnson et al., 2006; Takeda et al., 2003; Wang et al., 2005).

Activation of B cells, with the help of CD4+ T cells, is important for immune protection (Garenne and Lafon, 1998). When activated, primarily by the N protein of the rabies virus, CD4+ T cells produce specific cytokines (i.e. IL-4) that drive virus-neutralizing antibody (VNA) production. How antibodies mediate virus clearance is still unknown. One possible hypothesis is that VNA blocks virus attachment to host cells by complete masking of all G protein spikes of the rabies virion, which is termed as steric hindrance theory (Irie and Kawai, 2002) (Dietzschold et al., 1992). Another possible mechanism is VNA would induce conformational changes of the G protein spikes and finally causing abolishment of the receptor-binding ability of the virion (Irie and Kawai, 2002). The clearance of most viral infections is partly dependent upon destruction of the infected

cells, generally by CD8+ T cells (Iwasaki et al., 1977). Rabies-specific CD8+ T cells is the predominate immune response against rabies infection (Hooper, 2005; M, 2002).

The immune response is modulated according to the pathogenicity of rabies virus strains (Lafon, 2007). Pathogenic strains of rabies virus select immunosubversive strategies to escape the host immunity and cause acute encephalitis; while less pathogenic strains of rabies virus activate the innate immunity of the host which cause inflammation and adaptive immune response with T and B cells migrating into the nervous system. The immune response always is associated with neuronal destruction. Nonpathogenic viruses may be cleared away from the CNS due to little permanent neuron damage and appropriate immune response. Severe disease, such as irreversible paralysis or even death, may be caused by strong inflammation and an inappropriate immune response. Pathogenic RV evades the host immune response by not upregulating inflammatory chemokines and cytokines and decreasing infiltrating T and B cells. As a consequence, the virus invades the entire CNS causing death (Lafon, 2007).

Chemokine and rabies virus infection in CNS

Chemokines are a family of structurally related proteins that have similar functions. One of the major roles of the chemokine is to govern leukocyte movement across the areas of homeostasis, inflammation and development (Zlotnik et al., 1999). Chemokines may stimulate expression and activation of adhesion molecules on target leukocytes and promote their attachment to the vascular endothelium. Chemokines mediate extravasation of leukocytes from blood into the tissues by providing signals that convert the low affinity selectin-mediated interaction into the higher affinity integrin-mediated interaction on vascular endothelial cells. Once localized in the perivascular space, invading

leukocytes form perivascular cuffs and may further penetrate into the CNS parenchyma under the influence of chemokineg gradients (Glabinski and Ransohoff, 1999).

Viral infection of the CNS can result in expression of chemokines both by resident cells of the CNS as well as by inflammatory cells. In vitro studies have demonstrated that human microglia and astrocytes are able to express chemokine genes such as MCP-1, RANTES, and MIP-1α and β following infection with various viruses including measles virus and human coronavirus (Edwards et al., 2000; Vanguri and Farber, 1994; Xiao et al., 1998). Infection of rat astrocytes and microglia with paramyxovirus or Newcastle disease virus (NDV) result in rapid expression of RANTES and IP-10 (Fisher et al., 1995; Vanguri and Farber, 1994). Infection of mouse astrocytes with the mouse hepatitis virus (MHV) results in expression of chemokine genes MIP-2, IP-10, MCP1, MIP-1β, and MCP-3 (Lane et al., 1998).

Numerous studies have demonstrated a relationship between viral infection of the CNS, the expression of chemokine and leukocytes entering the CNS. In MHV infected mice, CD4+ and CD8+ T lymphocytes are required for efficient clearance of MHV from the CNS (Karpus et al., 1995; Parra et al., 1999; Piali et al., 1998). MIG and IP-10 attract activated T lymphocytes following binding to CXCR3. Treatment of MHV-infected mice with either anti-MIG or anti-IP-10 resulted in a significant decrease in numbers of CD4+ and CD8+ T lymphocytes infiltrating into the CNS (Farber, 1993; Farber, 1997; Liu et al., 2001; Vanguri and Farber, 1990). In WNV infected mice, IP-10 and receptor CXCR3 are required for T lymphocytes penetration into CNS (Klein et al., 2005; Zhang et al., 2008). CD3+ T cell infiltration is impaired in the CNS of IP-10 -/- or CXCR3-/- mice (Klein et al., 2005).

Recently, a gene array-based comparison of mouse brain gene expression in response to infection with different RV demonstrated that a number of chemokine genes are upregulated in mice infected with lab-attenuated RV (Wang et al., 2005). Proinflammatory chemokines including RANTES (CCL5), MCP1 (CCL2), MIP1-α (CCL3), IP-10 (CXCL10), and MIP1-β (CCL4) are all upregulated with some increased more than 100-fold in the CNS. MIP1-α and MIP1-β were only upregulated in mice infected with B2C but not SHBRV (Wang et al., 2005). Among some chemokines that were upregulated in both SHBRV and B2C-infected animals, the upregulation in mice infected with SHBRV is 2- to 20- fold lower than that in mice infected with B2C (Wang et al., 2005).

The previous results were supported by other researchers using lab-attenuated RV or similar virus to infect mice or neuronal cells (Jackson et al., 2006; Johnson et al., 2006; Nakamichi et al., 2004; Prehaud et al., 2005). Nakamichi et al reported that IP10 and RANTES were highly upregulated and other chemokines were not upregulated in RV-infected macrophages (Nakamichi et al., 2004). It is possible that CNS resident cells (e.g. microglial and astrocytes) and infiltrating inflammatory cells are responsible for the upregulation of so many chemokines in RV infection. RANTES and IP-10 transcripts were also observed upregulated in European bat lyssavirus (EBLV) infected mice brain (Mansfield et al., 2008). In a study involving a neuron cell line infected with RV (CVS strain), RANTES was found to be upregulated at 1 hour p.i., while some genes are upregulated later at 6 or 24 hours p.i. (Prehaud et al., 2005). Expression of chemokines including RANTES, MIP1-α, IP-10, and MIG (CXCL9) were strongly stimulated post RV infection. The transcriptions of IP-10, RANTES and other genes were increased by

IFN-α treatment (Prehaud et al., 2005). These observations led to the hypothesis that lab-attenuated RV is a potent inducer of chemokine expression, which might be an important mechanism of RV attenuation.

Blood brain barrier permeability changes during rabies virus infection

The blood brain barrier (BBB) is a membranic structure that acts primarily to protect the brain from chemicals from the blood, while still allowing essential metabolic function (Cserr and Knopf, 1992). It is composed of endothelial cells, which are packed very tightly in brain capillaries. This higher density restricts passage of substances from the bloodstream much more than endothelial cells in capillaries elsewhere in the body. Astrocytes surround the endothelial cells of the BBB, providing biochemical support to those cells.

The endothelial cells of capillaries in the brain are different to those found in peripheral tissues in various ways: 1) Brain endothelial cells are joined by tight junctions of high electrical resistance providing an effective barrier against molecules (Oldendorf WH, 1997). 2) Brain endothelial cells lack transendothelial fenestrations, and have high numbers of mitochondria (Reese TS, 1967). 3) In peripheral endothelial cells, there is good transcellular movement of molecules. There is no such movement in brain endothelial cells. 4) Brain capillaries are in contact with foot processes of astrocytes which essentially separate the capillaries from the neurons (Cserr and Knopf, 1992).

Recently, BBB has been suggested as playing an important role in RV infection and outcome. In one human rabies case, even in the absence of postexposure treatment (PEP), the victim naturally developed a high VNA titer and enhanced BBB permeability as evidenced by the appearance of serum proteins in the cerebrospinal fluid, and the

immune response to RV was sufficient to clear the infection from the CNS (Willoughby et al., 2005).

Since then many studies have been conducted, basically using laboratory adapted RV strains to investigate the mechanism of BBB opening. By using CVS-F3 (an antibody escape mutant derived from CVS virus (Irwin et al., 1999)) and SHBRV, Phares et al (Phares et al., 2006) and Roy et al (Phares et al., 2007) reported that the only difference is the change in the BBB permeability between mice infected with attenuated and wt RV. There is no difference in the level of viral antigen expression or the expression of inflammatory chemokines or cytokines. The BBB is opened when infected with lab-attenuated virus while infection with wt SHBRV does not induce changes in BBB permeability. Changes in BBB permeability allow immune effectors to cross the BBB and enter the CNS. Adoptive transfer of immune cells isolated from mice infected with CVS-F3 resulted in clearance of SHBRV from the CNS (Phares et al., 2007). They suggested that BBB opening is associated with clearance of the apathogenic RV from the CNS and failure to open the BBB leads to disease in SHBRV infection (Phares et al., 2007; Phares et al., 2006; Roy et al., 2007). The PLSJL mouse strain in which it is more easy to initiate CNS inflammation than the normal 129 mouse strain, was more resistant to the death caused by pathogenic RV infection (Roy and Hooper, 2008).

The mechanism leading to changes in BBB permeability after infection with laboratory-attenuated RV is not entirely clear. Roy et al hypothesized that TNF-a may contribute to alterations in BBB integrity since TNF-α can enhance the expression of ICAM-1 on endothelial cells (Phares et al., 2007). However, the BBB permeability and virus (CVS-F3) replication is very similar in WT and TNF-α-/- mice. In addition, the profile of

chemokine expression, inflammatory cell accumulation, and virus specific antibodies is similar in all the mice. The results indicate that expression of TNF-α is not directly required for opening of the BBB (Phares et al., 2007). Peroxynitrite ($ONOO^-$) has been reported to play a role in this process (Phares et al., 2007). Inhibition of peroxynitrite-dependent radicals by urate did not change the outcome of RV infection (Fabis et al., 2008). Thus it is not clear if peroxynitrite is the main trigger of BBB opening in RV infection. It is possible that the first small wave of lymphocytes may have another route to infiltrate into the CNS which is independent from increased BBB permeability. Then, the chemokines expressed by cells that have infiltrated the CNS can trigger the BBB opening.

Chemokines and BBB permeability changes

Various pathologic conditions involving inflammation of the CNS can lead to breakdown of the BBB. For many years, the directed migration of lymphocytes into tissue was ascribed to the activity of chemokines. Chemokines may stimulate expression and activation of adhesion molecules on target leukocytes and promote their attachment to the vascular endothelium. Once localized in the perivascular space, activated inflammatory cells may secrete more chemokines that can induce extravasation of additional blood-borne inflammatory cells. Invading leukocytes that form perivascular cuffs and may further penetrate into the CNS parenchyma under the influence of chemokine gradients (Glabinski and Ransohoff, 1999).

During CNS inflammation, chemokines may exert important functions during multiple steps of inflammatory cell extravasation. MCP-1 participates in recruiting leukocytes into the CNS. Evidence suggests that CCR2, a receptor for MCP-1, is present on brain

endothelial cells (Stamatovic et al., 2003). Stamatovic et al investigated a possible role for MCP-1 and CCR2 in breakdown of the BBB during CNS inflammation. MCP-1 decreased transepithelial electrical resistance and increased permeability to inulin across mBMEC monolayers from wild-type mice, but not across mBMEC monolayers from mutant mice that lacked CCR2 (Stamatovic et al., 2003). MCP-1 elicited rearrangement of the actin cytoskeleton, visible gaps between cells, and redistribution of tight junction proteins. Thus, MCP-1 appears to contribute to breakdown of the BBB during inflammation and may provide a therapeutic target to help limit injury to the CNS during inflammation (Stamatovic et al., 2003).

IP-10 recruits inflammatory cells (mainly T and NK cells) into the CNS crossing the BBB, and this function is intimately linked to BBB integrity. Treatment of mice with anti-IP-10 antibodies prevents recruitment of inflammatory cells into the CNS. Treatment of MHV-infected mice with anti-IP-10 resulted in a significant decrease in numbers of CD4+ and CD8+ T lymphocyte infiltrating into the CNS (Liu et al., 2001). IP-10 receptor CXCR3 deficient mice infected with dengue virus (Hsieh et al., 2006) or West Nile Virus (Zhang et al., 2008) had less effecter T cells recruited to the CNS.

MIP-1α may facilitate entry of inflammatory cells into the CNS by altering the integrity of the BBB and contribute to the pathogenesis of autoimmune and infectious diseases. Treatment of mice with anti- MIP1-α prevents recruitment of inflammatory cells into the CNS (Mucke and Eddleston, 1993; Tanabe et al., 1997). Man et al found that the interaction of MIP1-α and CCR5 triggered opening of endothelial tight junctions in the BBB (Man et al., 2007). Zozulya et al demonstrated that MIP1-α increases the

transmigration of dendritic cells across brain microvessel endothelial monolayer (Zozulya et al., 2007).

Goals and objectives

The main objective of this research is to investigate the induction of innate immune response in the PNS and CNS after infection with wt or attenuated rabies viruses and to correlate the expression of chemokines with infiltration of inflammatory cells into the CNS, changes in BBB integrity, and the outcome in RV-infected animals.

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CHAPTER 2

ROLE OF CHEMOKINES IN THE ENHANCEMENT OF BBB PERMEABILITY AND INFILTRATION OF INFLAMMATORY CELLS AFTER RABIES VIRUS INFECTION

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[1]Yi Kuang, Sarah N Lackay, Ling Zhao, and Zhen F Fu
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Abstract

Induction of innate immunity, particularly interferon and chemokines, by rabies virus (RV) infection has been reported to be inversely correlated with pathogenicity. To further investigate the association between the expression of chemokines and RV infection, laboratory-attenuated RV (B2C) and wild-type (wt) RV (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 RV induces chemokines expression 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 RV.

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 (Fu, 1997). In the United States, dog rabies has almost been eradicated through massive vaccination during the past 6 decades (Krebs et al., 1996; Noah et al., 1998). However, bat (particularly the silver-haired bats) 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 almost always fatal (Fu, 1997). Despite the lethality of rabies, only mild inflammation and little neuronal destruction are observed in the CNS of rabies patients (Miyamoto and Matsumoto, 1967; Murphy, 1977). On the other hand, laboratory-attenuated RV induces extensive inflammation and neuronal degeneration in experimentally infected animals (Miyamoto and Matsumoto, 1967; Murphy, 1977). To understand the differential effects, mice were infected with two rabies virus (RV) strains, one laboratory-attenuated RV and the other wildtype (wt) RV, and compared the host responses to infection (Wang et al., 2005). It was found that laboratory-attenuated RV induced extensive inflammation, apoptosis, and neuronal degeneration in the CNS; however, wt RV caused little or no neuronal damage. Furthermore, laboratory-attenuated RV 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 RV (Wang et al., 2005). The induction of innate immunity has been confirmed when other laboratory-attenuated viruses were used 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 RV is a potent inducer of host innate immunity, which might be an important mechanism of RV attenuation (Wang et al., 2005).

This hypothesis has recently been challenged. By using CVS-F3, an antibody escape mutant derived from CVS virus (Irwin et al., 1999) and SHBRV, a wt RV isolated from a human patient (Rupprecht et al., 1997), it has been shown that the major difference is the change in blood-brain barrier (BBB) permeability between mice infected with attenuated- and wt RV (Fabis et al., 2008; Phares et al., 2007; Roy and Hooper, 2007; Roy et al., 2007). The BBB is more permeable in mice when infected with laboratory-attenuated RV than in mice infected with wt RV. Enhancement of BBB permeability allows immune effectors to cross the BBB and enter the CNS. Adoptive transfer of immune cells isolated from mice infected with laboratory-attenuated RV resulted in clearance of wt RV from the CNS (Roy et al., 2007). These studies suggest that changes in BBB permeability are associated with clearance of the apathogenic RV from the CNS (Phares et al., 2007; Roy et al., 2007). Failure to open the BBB leads to disease in wt RV-infected mice.

This study attempts to determine the contributions of the innate immune response, especially inflammatory chemokines, in the enhancement of BBB permeability and the outcome of RV infection. If increased BBB permeability is the major contributor towards survival, what is the mechanism that is responsible for this change in RV-infected animals? Therefore, expression of chemokines, inflammatory responses, and changes in

BBB permeability were investigated in mice infected with laboratory-adapted or wt RV. It was found that expression of chemokines was closely associated with infiltration of inflammatory cells and increases in BBB permeability. Among the chemokines, the expression of IP-10 was best correlated with such changes in mice infected with laboratory-attenuated RV. These data indicated that laboratory-attenuated RV 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 and attenuation of RV virulence.

2. Materials and methods

2.1. Viruses, antibodies

Four viruses were used in this study and they are SHBRV, DRV, B2C, and SN-10. SHBRV is a wt RV isolated from a human patient and this virus strain has been associated with human rabies in the Unites States for the past two decades (Faber et al., 2004; Rupprecht et al., 1997). DRV is a virus isolated from dogs (Dietzschold et al., 2000). B2C is a laboratory-adapted and attenuated virus isolated from CVS-24 by passaging in BHK cells (Morimoto et al., 1998). SN-10 is a cloned virus derived from the 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^{o}C. Fluorescein isothiocyanate (FITC)-conjugated antibody against the RV N protein was purchased from FujiRebio (FujiRebio Diagnostic INC, PA). Anti-RV nucleoprotein (N) monoclonal antibody 802-2 (Hamir et al., 1995) was obtained from Dr. Charles Rupprecht, Center for Disease Control and Prevention (CDC). Anti-RV glycoprotein (G) polyclonal antibody was prepared in rabbit 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 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 SYBR Green PCR and 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 method 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 were listed in a previous paper (Wang et al., 2005). To determine the virus replication and transcription in each sample, virus specific nucleoprotein mRNA and genomic RNA were measured by quantitative RT-PCR. Synthetic cDNA standards were made from RV N gene. Standard curves constructed with Ct value obtained using dilutions of these standards. The mRNA and genomic RNA copy numbers in each sample were normalized to the copy number of the standard curve respectively.

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 to 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/10g body weight and then perfused by intracardiac injection of PBS followed by 10% neutral buffered formalin as described previously (Li et al., 2005). Brain tissues 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-paraffin, slides were stained with hematoxylin and eosin (H&E).Slides were also heated in antigen unmasking solution (Vector Laboratories, CA) above 90°C for 20 min and naturally cooled down to room temperature. Anti-RV nucleoprotein monoclonal antibody 802-2 was used to detect the viral antigen. The primary antibody and then secondary antibodies (biotinylated) were used for immunological reaction as described (Yan et al., 2001). The avidin-biotin-peroxidase complex (Vector Laboratories,CA) was then used to

localize the biotinylated antibody. Finally, diaminobenzidine (DAB) was used as a substrate for color development. The intensity of DAB signals corresponding to RV N or G antigen were measured by Image-pro Plus software.

2.6. Enzyme-linked immunosorbent assay (ELISA)

ELISA was used to quantify the amount of MIP-1α, IP-10 and RANTES in mouse brain suspensions by using the murine ELISA Kit (R&D Systems, Minneapolis, MN) according to the manufacture's protocol.

3. Result

3.1. Differential induction of chemokines by laboratory-attenuated and wt RV

Previously it has been shown that lab-attenuated B2C activated innate immunity in mouse CNS while wt SHBRV did so to a much less degree (Wang et al., 2005). To extend these studies, groups of ICR mice were infected with more wt RV (SHBRV or DRV, a dog-derived wt RV) or laboratory-attenuated RV (B2C or SN-10) by the intracerebral (IC) route with virus dose of 10 $ICLD_{50}$. At the time of severe paralysis, mice were sacrificed and brains were removed for immunohistochemistry to quantitate the N expression and realtime-PCR to quantitate innate immunity genes expression. The viral N gene level was measured by the intensity of DAB signals by Image-pro Plus Software. As shown in Fig 1, the levels of N expression were similar in mice infected with each virus, indicating that the level of viral replication in the CNS is similar for all the viruses. On the other hand, significantly more G protein was detected in mice infected with laboratory-attenuated virus than with wt RV, an observation similar to those reported previously (Sarmento et al., 2005; Wang et al., 2005). To determine the expression level of genes relevant to the innate immunity, real-time PCR was used to measure the expression of chemokine genes. As shown in Table 1, the expression of these genes is up-regulated after RV infection; however, the upregulation is usually higher in mice infected with laboratory-attenuated RV than wt RV. MCP1, MIP1β, RANTES, MCP3, Mig, IP-10 are more upregulated in B2C and SN-10 infected mice than in DRV and SHBRV infected mice. Most of these genes are upregulated 2 to 1000 fold more in B2C- than in DRV-infected mice or 2 to 20 fold more than in SHBRV-infected mice. The upregulation of these genes in mice infected with SN-10 are 2 to 50 fold or 2 to 61 fold higher than that in mice infected with

DRV or SHBRV, respectively. The most up-regulated gene is IP-10, which is increased 2394-fold in animals infected with B2C. These data are consistent with previous findings (Wang et al., 2005) and suggest that differential induction of chemokines by wt RV and laboratory-attenuated RV may contribute to RV pathogenicity and/or attenuation.

3.2. Evaluation of viral antigen and viral RNA in mice after IM infection with laboratory-attenuated and wt RV

Since most of the RV infections are by animal bites, wt DRV and laboratory-attenuated B2C each at 10 $IMLD_{50}$ were intramuscularly (IM) inoculated into Balb/c mice. At days 3, 6 and 9 p.i., animals were sacrificed and brains, spinal cords, as well as dorsal root ganglions (DRG) were collected for evaluation of viral antigen by immunohistochemistry and/or viral RNA by realtime-PCR. Viral antigen was detected in the DRG in most of the infected animals at day 3 and in all the infected animals by day 6 p.i. although the level of viral antigen was low (Fig 2A). By day 9 p.i., high level of viral antigen was detected in the DRG, particularly in B2C-infected mice (Fig 2A). Virus antigen could not be detected in either the spinal cord or the brain infected with DRV or B2C at day 3 p.i. At days 6 and 9 p.i., virus antigen was only detected sparsely in the spinal cord and brain in B2C- or DRV-infected mice (Fig. 2A). Thus viral replication (mRNA and genomic RNA) in the CNS was evaluated by real-time PCR using RV gene-specific primers. As show in Fig 2B, more mRNA and genomic RNA were detected in the CNS (spinal cord and brain) of mice infected with B2C than DRV at day 6 p.i. On the other hand, more mRNA and genomic RNA were detected in the CNS of mice infected with DRV than B2C at day 9 p.i.

3.3. Changes in BBB permeability in mice after IM infection with laboratory-attenuated and wt RV

To investigate if infection with each RV induces changes in BBB permeability, the leakage of the sodium fluorescein (NaF) from the circulation into the CNS tissues was measured in the spinal cord, cerebellum, and cerebrum of mice infected with 10 $IMLD_{50}$ of each virus. As shown in Fig3A, no significant change in BBB permeability was observed in mice infected with either virus at day 3 p.i.. BBB permeability was significantly enhanced in all brain regions of the CNS by 6 days after infection with B2C, but not with DRV when compared to sham-infected mice. By day 9 p.i., BBB permeability in mice infected with B2C returned to the level as seen in DRV-infected mice, which is not significantly different from sham-infected mice. To ensure that the changes in BBB permeability are not effected by the virus concentration, mice were infected with increasing dose of DRV or decreasing dose of B2C. As shown in Fig3B, increasing DRV dose to 100$IMLD_{50}$ did not result in enhanced BBB permeability in brain while decreasing B2C dose to 1$IMLD_{50}$ led to enhanced BBB permeability. At day 6 p.i., mice infected with 1$IMLD_{50}$ B2C show significant higher BBB permeability changes than mice infected with either 10 or 100 $IMLD_{50}$ DRV. These data indicate that enhancement of BBB permeability is not dose-dependent, but virus-dependent.

3.4. Induction of inflammatory chemokines in mice after IM infection with laboratory-attenuated and wt RV

To determine the innate immune responses following RV infection, spinal cords and brains from mice infected with 10$IMLD_{50}$ of DRV or B2C were collected on day 3, 6 and 9 p.i. and homogenates were subsequently assayed for the expression of cytokines and

chemokines by real-time PCR. At day 3 p.i., the expression of TLR-3, IRF-7, IFNα4, IFNγ, IL-6, and Mx-1 was upregulated in the spinal cord, while up-regulation in the expression of TLR3, IRF7, IFNα4, IFNα5, IFNγ, IL-6, MMP9, Mx-1,and OAS-1g was observed in the brain of mice infected with B2C (data not shown). None of these genes was found to be up-regulated in either the spinal cord or the brain of mice infected with DRV. By 6 days p.i., expression of cytokines and chemokines was highly increased in the CNS of mice infected with B2C (Figs. 4A). The most up-regulated gene in expression is IP-10 in the spinal cord (data not shown) and MIP-1α in the brain when compared to sham-infected mice. In mice infected with DRV, expression of these chemokines genes were either not changed when compared to sham-infected mice or up-regulated to a much lesser extent than that observed in B2C-infected animals. These data are consistent with previous findings (Wang et al., 2005) and suggest that laboratory-attenuated RV activates the expression of the genes involved in the innate immunity while wt RV does not. Furthermore, the expression pattern of IP-10, MIP-1a, and RANTES are parallel to the changes in BBB permeability (Figs. 4A). These data suggest that expression of these chemokines may play an important role in the enhancement of BBB permeability. The expression of TNF-α in the brain is also parallel to the changes in BBB permeability; however, it has been reported that TNF-α is not a determinant of changes in BBB integrity (Phares et al., 2007).

Since the most-upregulated genes are chemokines, particularly IP-10, RANTES, and MIP-1α, ELISA was carried out to determine protein expression of the most-upregulated chemokine genes, IP-10, RANTES, and MIP-1α, in brains mice infected with 10IMLD_{50} of B2C or DRV. As shown in Fig. 4B, the expression of IP-10 MIP-1α, and RANTES

was significantly upregulated at day 6 p.i. in the brains of mice infected with B2C, correlating with the enhancement of BBB permeability. On the other hand, in mice infected with DRV, IP-10, MIP-1α, and RANTES levels were similar to uninfected mice at all three time points. The expression patterns of IP-10 and MIP-1α in the brain were parallel to those observed in the expression of mRNA and in the changes in BBB permeability. The expression of RANTES increased at days 6 and reached peak by day 9 p.i., however there is no significant difference between B2C- and DRV-infected mice at day 9 p.i.. The most increased level of protein expression is IP-10 with expression of 400 pg per mg of mouse brain. Thus, the data suggest that differential induction of chemokines, particularly IP-10, by B2C and DRV may contribute to the observed differences in innate immune responses and BBB permeability changes.

3.5. Inflammatory response in mice after IM infection with laboratory-attenuated and wt RV

To investigate if upregulation of chemokine expression led to infiltration of inflammatory cells into the infected nervous system, DRGs, spinal cords and brains were collected for histopathology and immunohistochemistry. At day 3 p.i., infiltration of inflammatory cells into the CNS was minimal in mice infected with either virus (Fig 5 A). Severe inflammation was observed in the spinal cord and brain of mice infected with B2C at 6 days p.i., and was found subsided by day 9 p.i. Only mild inflammation was observed in DRV-infected mice. To qauntitate the inflammatory response in the CNS, CD3-positive cells were measured by using anti-CD3 antibodies in the thalamus/hypothalamus. As shown in Fig. 5B-C, no significant difference in the number of CD3-positive cells was found between B2C-, DRV- or mock-infected mice at day 3 p.i. By day 6 p.i.,

significantly more CD3-positive cells were found in B2C- than in DRV- or mock-infected mice. By day 9 p.i., the number of CD3-positive cells in B2C-infected mice declined, but was still more than that found in DRV-infected mice (Fig 5B and C). The patterns of CD3-positive cells in DRG or spinal cord are similar to those in the brain. The results thus suggested that laboratory-attenuated RV induces more inflammation than wt RV.

4. Discussion

Previously we showed that laboratory-attenuated RV is a potent inducer of innate immune response while wt RV is not, which led us to hypothesize that induction of innate immunity is an important mechanism of RV attenuation (Wang et al., 2005). Recently, it has been reported that changes in BBB permeability is important in virus clearance and thus plays a decisive role in virus attenuation (Phares et al., 2007; Roy and Hooper, 2007; Roy et al., 2007). In this study, we attempted to determine if induction of innate immunity, especially chemokines, is related to, or associated with, the changes in BBB permeability. Our results suggest that these two events are closely related, at least as a function of time after RV infection.

Our previous studies with microarray analysis and real-time PCR showed that attenuated B2C induced higher levels of chemokines expression than wt SHBRV (Wang et al., 2005). In the present study, we extended our previous study by including more laboratory-attenuated and wt viruses. It was found that laboratory-attenuated RVs (B2C and SN-10) consistently induced higher level of expression of chemokines genes than wt viruses (SHBRV and DRV) irrespective the route of infection (IC vs. IM) (Table 1). Furthermore, infection with attenuated RV as well as RV-like viruses upregulate chemokines expression (Johnson et al., 2006; Mansfield et al., 2008; Nakamichi et al., 2004; Prehaud et al., 2005). Together, these studies further confirm that infection with laboratory-attenuated RV induces the expression of genes involved in innate immunity, particularly chemokines, which may be one of the mechanisms by which RV is attenuated.

A series of recent studies in Hooper's laboratory showed that changes in BBB permeability are associated with clearance of the apathogenic RV isolates (CVS-F3, ERA, and PM) from the CNS, while pathogenic RV strains (DRV, Skunk RV etc) fail to change BBB permeability and deliver immune effectors into CNS (Phares et al., 2007; Roy and Hooper, 2008; Roy et al., 2007). Adoptive transfer of immune cells isolated from mice infected with attenuated RV resulted in clearance of wt RV from the CNS (Roy et al., 2007). Enhancement of the BBB permeability with myelin basic protein (MBP) can also increase the survivorship of mice after infection with wt RV (Roy and Hooper, 2007). The PLSJL mice strain in which is easier to initiate CNS inflammation than the normal 129 mice strain was more resistant to the death of pathogenic RV infection (Roy and Hooper, 2008). All these studies indicate that failure to open the BBB leads to disease in RV infections. However, the mechanism leading to changes in BBB permeability after infection with laboratory-attenuated RV is not entirely clear. TNF-α, an inflammatory cytokine, has not been found to be important in enhancing BBB permeability although it is upregulated after infection with laboratory-attenuated RV (Phares et al., 2007). Peroxynitrite ($ONOO^-$) has been reported to play a role in this process (Phares et al., 2007). Inhibition of peroxynitrite-dependent radicals by urate did not change the outcome of RV infection (Fabis et al., 2008). Thus it is not clear if peroxynitrite is the main trigger of BBB opening in RV infection.

In this study, an attempt was made to determine the contribution of chemokines in the enhancement of BBB permeability and the outcome of RV infection. Two viruses (wt DRV and laboratory-attenuated B2C) were used to infect Balb/c mice by IM route and the induction of chemokines and changes in BBB permeability were monitored. It was

found that laboratory-attenuated B2C induced the expression of many chemokines, particularly at day 6 p.i. In addition, infiltration of inflammatory cells was highest at this time of infection. Enhancement of BBB permeability in the CNS was found only in mice infected with B2C at day 6 p.i. Therefore the induction of inflammatory chemokines correlates with the changes in BBB permeability at least as a function of time post infection. Detailed analysis indicates that upregulation of chemokine genes and the increased protein expression correlates with changes in BBB permeability in B2C-infected mice. Among these, the upregulation of IP-10 was the highest and the timing of IP-10 upregulation parallel to the enhancement of BBB permeability. Chemokines play a major role in changes of BBB permeability (Glabinski and Ransohoff, 1999; Man et al., 2007; Zozulya et al., 2007). Increased expression of Mig, IP-10 and RANTES are present within the cerebral spinal fluid (CSF) and CNS tissue of MS (multiple sclerosis) patients during periods of clinical attack and correlated with infiltration of leukocytes into the CNS (Sorensen et al., 1999). Administration of rabbit antisera specific for either Mig (CXCL9) or IP-10 to mouse hepatitis virus (MHV) infected mice correlated with a dramatic decrease of infiltrating cells (Liu et al., 2001). Man et al (2007) found that the interaction of MIP-1α and CCR5 (MIP1α receptor) triggered opening of endothelial tight junctions in the BBB. Zozulya et al (2007) demonstrated that MIP-1α increases the transmigration of dendritic cells across brain microvessel endothelial monolayer. Thus upregulated expression of chemokines, particularly IP-10, may be responsible for the enhancement of BBB after infection with attenuated RV.

Expression of IP-10 within the brain is often associated with increased infiltrating cells in MHV- (Klein, 2004) or herpes simplex virus (HSV-) (Molesworth-Kenyon et al., 2005)

infected animals. Treatment of mice with anti-IP-10 antibodies prevents recruitment of inflammatory cells into the CNS. Treatment of MHV-infected mice with anti-IP-10 resulted in a significant decrease in numbers of CD4+ and CD8+ T lymphocytes infiltrating into the CNS (Liu et al., 2001). IP-10 receptor CXCR3 deficient mice infected with Dengue virus (Hsieh et al., 2006) or West Nile Virus (Zhang et al., 2008) exhibited less effecter T cells recruited to the CNS. IP-10 recruits inflammatory cells (mainly T and NK cells) into the CNS crossing BBB, and this function is intimately linked to BBB integrity. In our study, increased expression of IP-10 correlates with the enhancement of BBB permeability as a function of time in mice infected with laboratory-attenuated RV, suggesting that IP-10 may be responsible for the increased infiltration of inflammatory cells into the CNS, which in turn enhances the BBB permeability.

In conclusion, the results from the present study confirm our previous studies that laboratory-attenuated RV is more active in inducing innate immune responses than wt RV. Overall the induction of innate immunity, particularly the up-regulation of chemokines and infiltration of inflammatory cells in to the CNS, correlates with the enhancement of BBB permeability in mice infected with laboratory-attenuated B2C. It is possible that laboratory-attenuated RV induces the expression of these chemokines which leads to infiltration of inflammatory cells into the CNS. Infiltration of inflammatory cells enhances the BBB permeability, ultimately allowing immune effectors into the CNS and clearing the infected virus. Among these chemokines, IP-10 is the mostly upregulated chemokine and its upregulation correlates closely with the enhancement of BBB permeability. Thus it is possible that expression of IP-10 (to a lesser extent, MIP-1α) plays a major role in the enhancement of BBB permeability. Further studies are

warranted to confirm such findings, for example, in knockout mice or in mice treated with anti-chemokine antibodies or siRNA.

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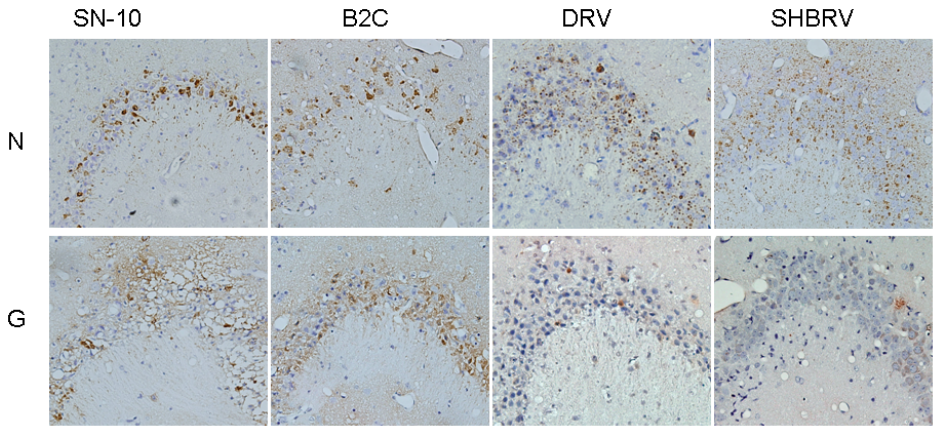
Table 2.1

Expression of inflammatory genes in mice brain infected with RV.

	Realtime-PCR (Fold change)			
	DRV	**SHBRV**	**B2C**	**SN-10**
MCP1 (CCL2)	**8.5**	**4.6**	**11**	**283**
MIP1α (CCL3)	**50.5**	**61**	**44**	**702**
MIP1β (CCL4)	**5.8**	**13**	**15.4**	**67.6**
RANTES (CCL5)	**17.9**	**267**	**413**	**305**
MCP3 (CCL7)	**18.4**	**101**	**226**	**242**
MCP5 (CCL12)	**2.6**	**158**	**155**	**82.2**
Mig (CXCL9)	**3.0**	**2.4**	**43.1**	**24.7**
IP-10 (CXCL10)	**2.5**	**819**	**2394**	**56.6**

(A)



(B)

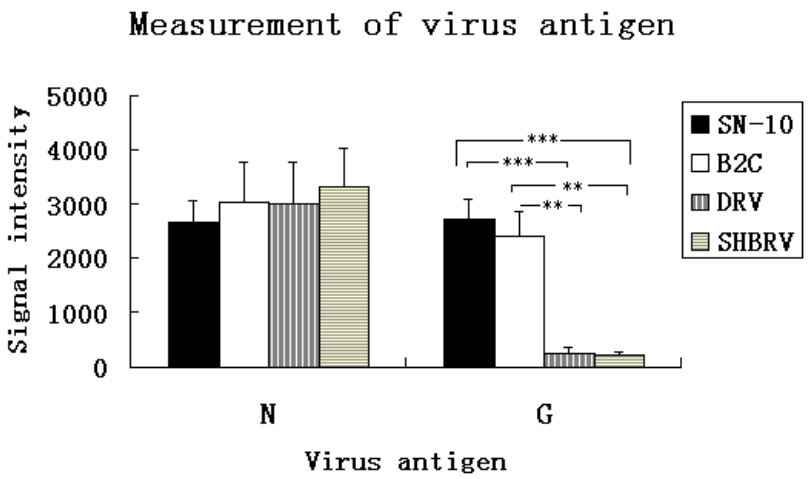
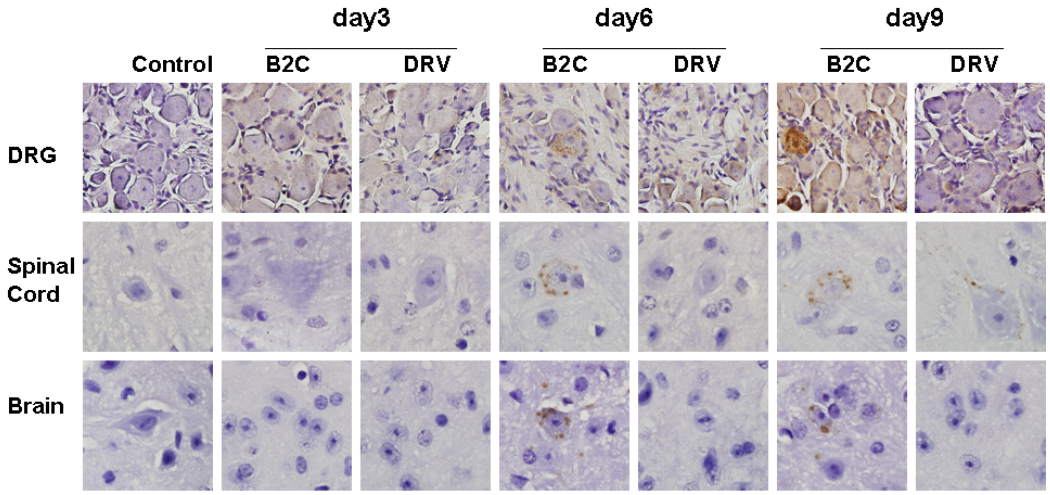


Figure 2.1. Detection of viral antigen in mice infected with different RVs. Viral antigen including both RV-N and RV-G were detected by immunohistochemistry (A). The intensity of DAB signals corresponding to viral antigen was measured in the hippocampus area of 3 animals by Image-pro plus software. The average of signal intensity and standard deviation are shown (B). Statistically significant differences between the viruses infected mice were determined by the Mann-Whitney *U* test, *** (p<0.0001) and ** (p<0.01). Magnificant 20×.

(A)



(B)

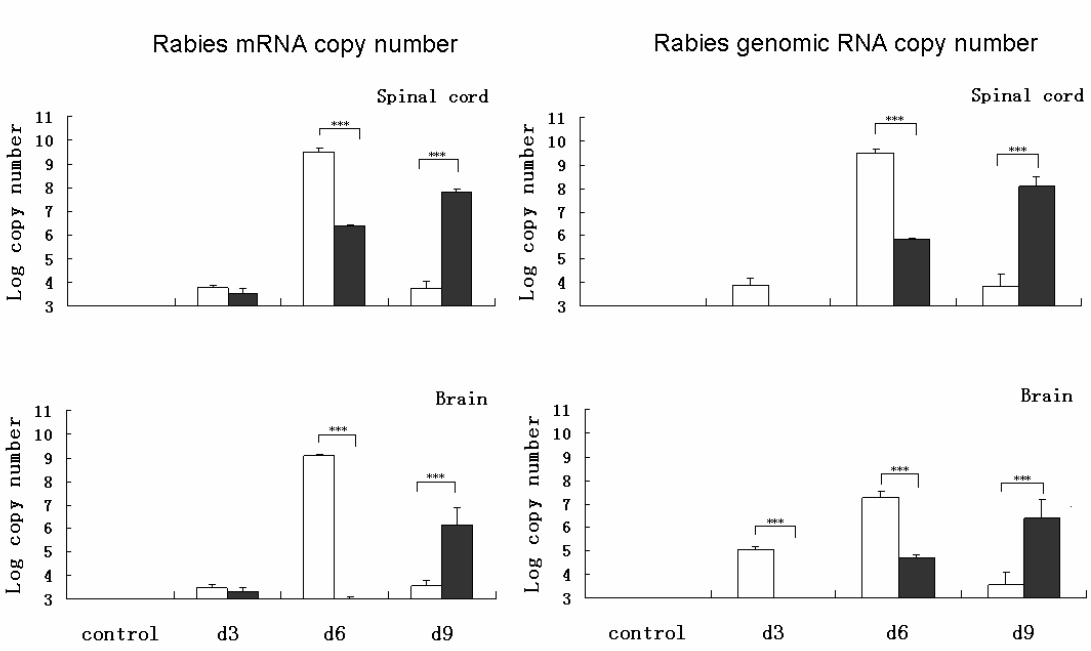
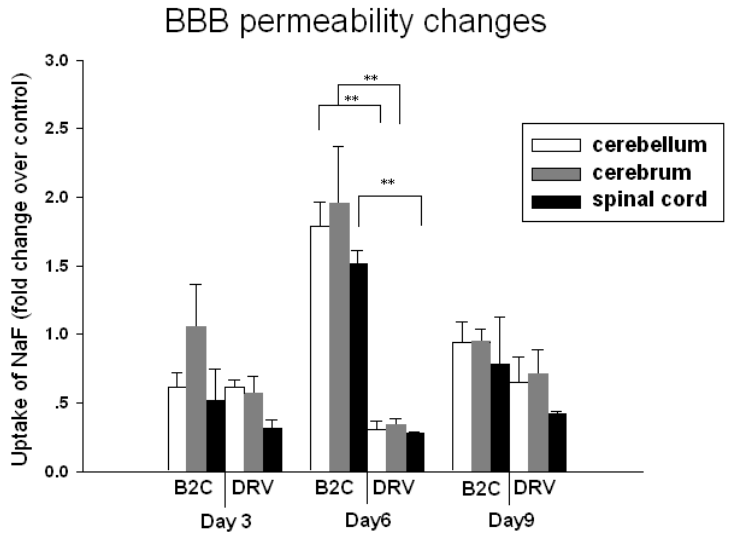


Figure 2.2. Detection of viral antigen in mice infected with B2C or DRV at different time points with 10 $IMLD_{50}$. RV N protein was detected by immunohistochemistry in mouse DRG, spinal cord and brain section (A). By using viral gene-specific primers, RV mRNA and genomic RNA were detected in B2C (white histogram) and DRV (black histogram) infected mice (C). Statistically significant differences between the infected with uninfected mice were determined by the Mann-Whitney *U* test, *** (p<0.0001), ** (p<0.001), * (p<0.005), and · (p<0.01). Magnificant 40× in DRG, 100× in spinal cord and brain (N antigen staining).

(A)



(B)

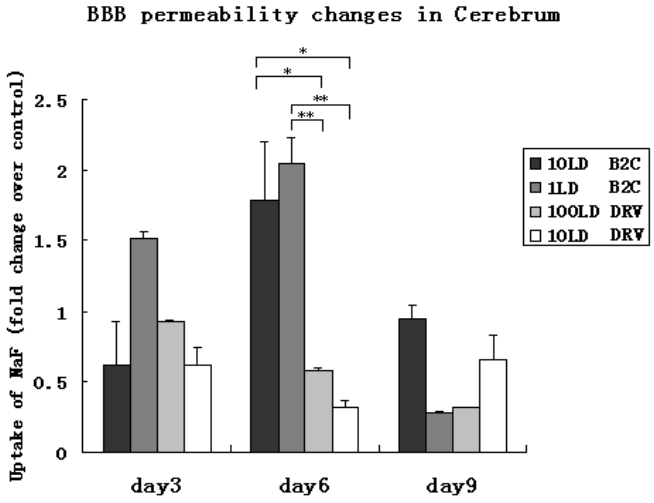


Figure 2.3. BBB permeability in RV infected mice. BBB permeability changes are presented as the fold increase in Na-fluorescein (Na-F) uptake in the cerebellum, cerebrum, and spinal cord with the levels in 10IMLD_{50} infected mice over the level of Na-F in uninfected mice (A). BBB permeability changes are also detected in different virus inoculate dose (B). Statistically significant differences between the infected with uninfected mice were determined by the Mann-Whitney *U* test, ** (p<0.01).

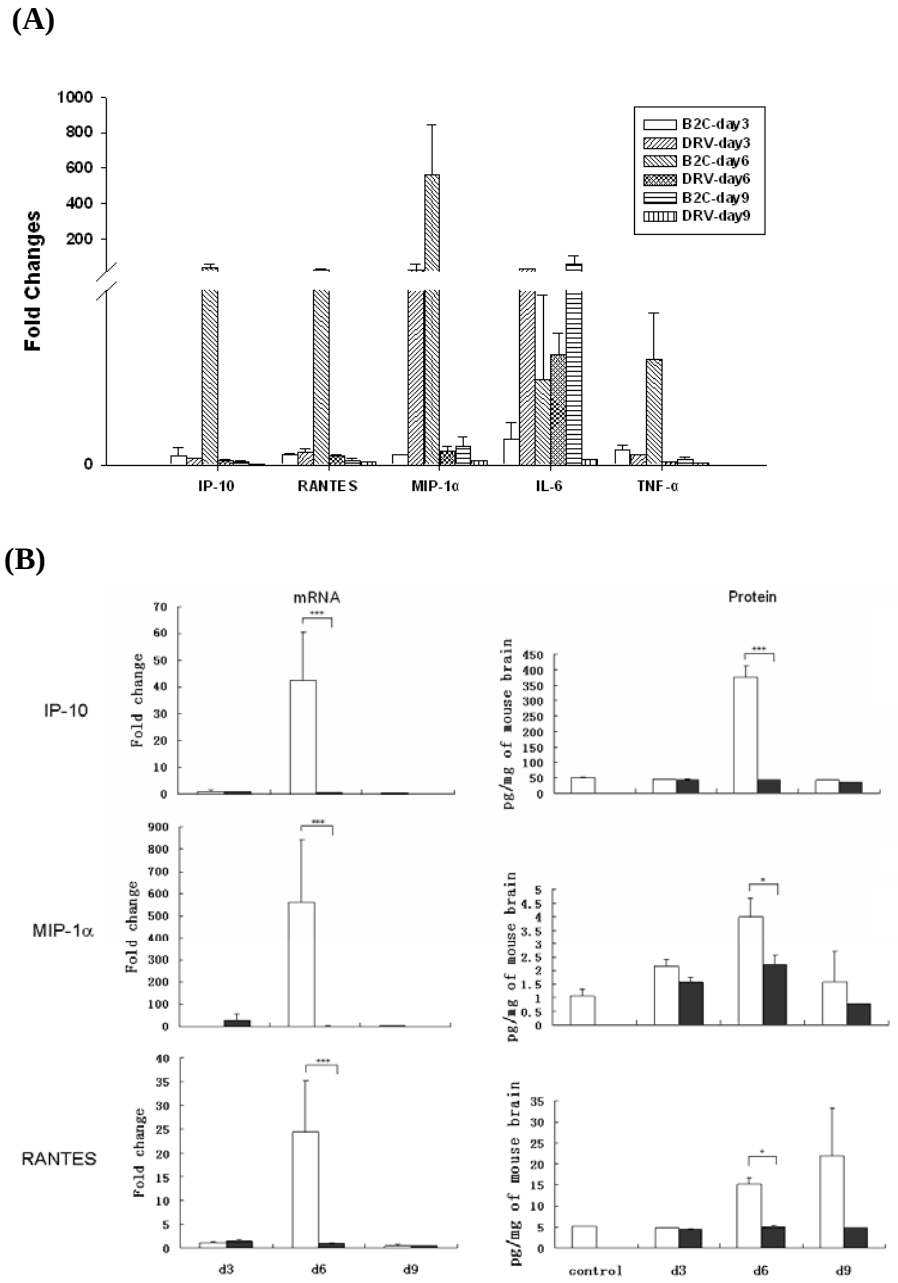


Figure 2.4. Determination of proinflammatory cytokine and chemokine levels in RV infected mice. Balb/c mice were infected with 10 $IMLD_{50}$ of B2C or DRV. Chemokine and cytokine levels in brain (A) were assayed by realtime PCR as described in material and methods. The mRNA data are expressed as the mean ± SEM of specific genes mRNA over the housekeeping gene mRNA in infected mice tissue minus the background levels from uninfected mice tissue. IP-10, MIP-1α, and RANTES mRNA (left) or protein level (right) were assayed by realtime PCR or ELISA as described in material and methods (C). Mice infected with B2C or DRV was shown in white or black histogram, respectively.

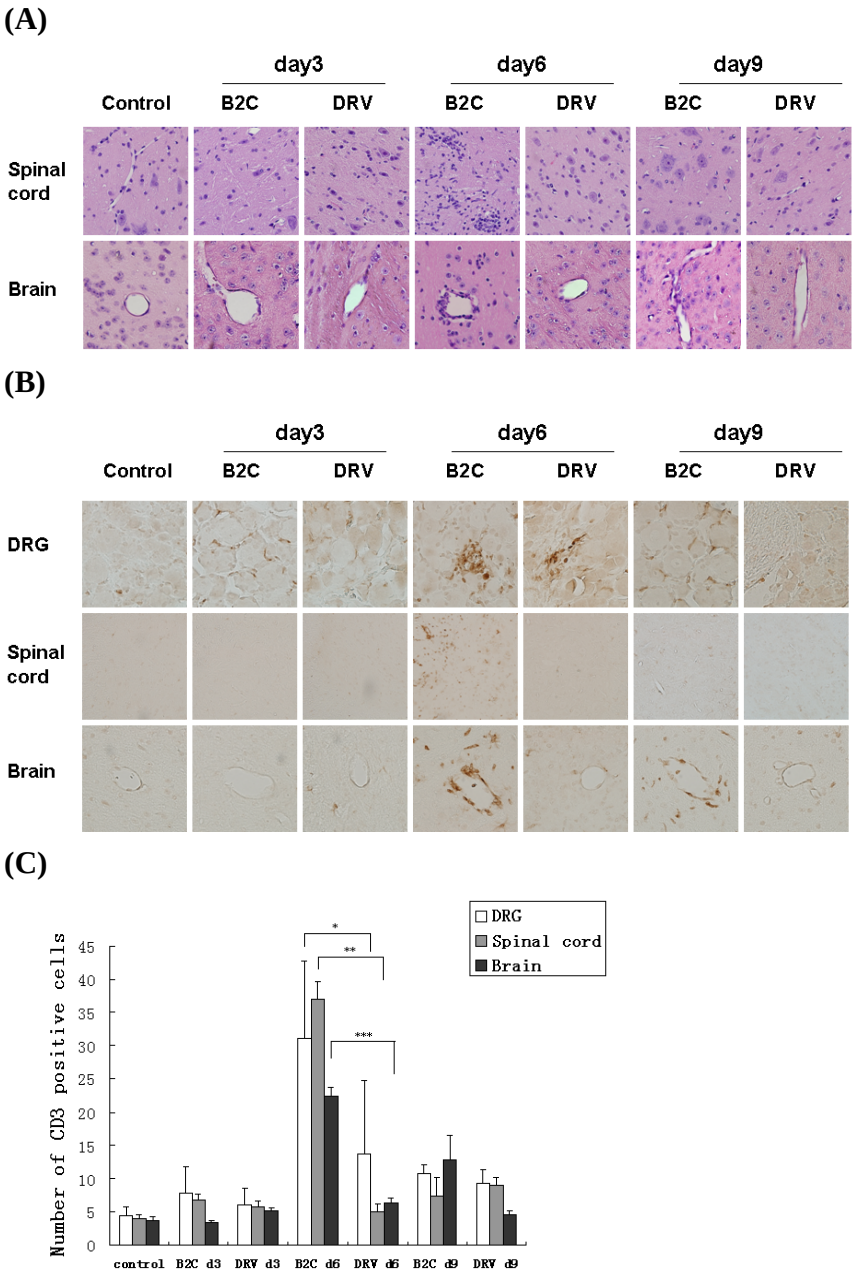


Figure 2.5. Detection of inflammation and CD3-positive cells in the mouse nervous tissue. Mice were infected with B2C or DRV with 10IMLD$_{50}$ and were transcardially perfused with 10% formalin at days 3, 6, or 9 p.i. Paraffin sections were subjected to HE staining (A) or immunohistochemistry for detecting CD3-positive cells (B). Three serial sections and two vessels were selected from each mouse for quantification, and the average numbers of CD3-positive cells were obtained for statistical analysis (C). Statistically significant differences between the infected with uninfected mice were determined by the Mann-Whitney *U* test, as *** (p<0.0001), ** (p<0.01), * (p<0.05), and · (p<0.1). Magnificant 20× in the DRG and spinal cord sections, 40× in the brain.

CHAPTER 3

Conclusions

There are significant differences between different strains of virus in their ability to infect, spread within the body, and produce disease (Miyamoto and Matsumoto, 1967). Laboratory strains of "fixed" virus have low pathogenicity when inoculated peripherally in low doses and are used to produce vaccines, or employed in diagnostic and research procedures. Wt RV is virulent and lethal, despite the fact it causes very little neuronal pathology or damage in the CNS (Miyamoto and Matsumoto, 1967; Murphy, 1977).

Previous studies have shown that infection of the CNS with laboratory-attenuated virus and wt RV induce different host immune responses. Laboratory-attenuated RV induces extensive inflammation and neuronal degeneration in experimental animals (Sarmento et al., 2005; Wang et al., 2005). Wt RV causes very little neuronal pathology or damage in the CNS of rabies patients and animals(Murphy, 1977). Our study extended previous studies by including more laboratory-attenuated and wt viruses. It was found that laboratory-attenuated RVs (B2C and SN-10) consistently induced higher level of expression of chemokines genes than wt viruses (SHBRV and DRV), irrespective the route of infection. Our results thus confirm previous findings that lab attenuated RV induces while wt RV evades innate immune responses in the CNS.

Recently, the BBB has been suggested as playing an important role in RV infection and outcome. BBB permeability is enhanced when mice are infected with lab-attenuated virus

(CVS-F3) while infection with wt SHBRV does not induce changes in BBB permeability (Roy and Hooper, 2007; Roy et al., 2007). Similar to previous studies, enhancement of BBB permeability in the CNS was found only in mice infected with B2C at day 6 after infection.

Chemokine expression, inflammatory responses, and change in BBB permeability were investigated in mice infected with attenuated B2C and wt DRV. The expression of chemokines was paralleled by infiltration of inflammatory cells and increased BBB permeability. The timing of chemokine (especially IP-10) expression, BBB permeability changes, and inflammation in the CNS suggest that the in situ production of IP-10 may be more important in infiltration of circulating leukocytes and BBB opening than other chemokines.

The association of expression of chemokines (especially IP-10) within the brain and cell infiltration is evident from previous studies. IP-10 is often correlated with increased infiltrating cells in MHV (Klein, 2004) or herpes simplex virus (HSV) (Molesworth-Kenyon et al., 2005) infected animals. Treatment of MHV-infected mice with anti-IP-10 results in a significant decreased in numbers of CD4+ and CD8+ T lymphocytes infiltrating into the CNS (Liu et al., 2001).

Over all our study suggested that chemokines, especially IP-10, may be responsible for the increased infiltration of inflammatory cells into the CNS, which in turn enhances BBB permeability.

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