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Immune evasion by rabies viruses through the maintenance of blood-brain barrier integrity

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The attenuated rabies virus (RV) strain Challenge Virus Standard (CVS)-F3 and a highly pathogenic strain associated with the silver-haired bats (SHBRV) can both be cleared from the central nervous system (CNS) tissues by appropriate antiviral immune mechanisms if the effectors are provided access across the blood-brain barrier (BBB). In the case of SHBRV infection, antiviral immunity develops normally in the periphery but fails to open the BBB, generally resulting in a lethal outcome. To determine whether or not an absence in the CNS targeted immune response is associated with the infection with other pathogenic RV strains, we have assessed the development of immunity, BBB permeability, and immune cell infiltration into the CNS tissues of mice infected with a variety of RV strains, including the dog variants responsible for the majority of human rabies cases. We demonstrate that the lethal outcomes of infection with a variety of known pathogenic RV strains are indeed associated with the inability to deliver immune effectors across the BBB. Survival from infection with certain of these viruses is improved in mice prone to CNS inflammation. The results suggest that competition between the activity of the immune effectors reaching CNS tissues and the inherent pathological attributes of the virus dictates the outcome and that intervention to deliver RV-specific immune effectors into CNS tissues may have general therapeutic value in rabies. *Journal of NeuroVirology* (2008) **14,** 401–411.

Keywords: blood brain barrier; CNS immunity; immune evasion; rabies virus

Introduction

Rabies has been known for centuries as a deadly neurological disease of both humans and animals. The causative agent belongs to a group of antigenically related viruses named after the disease, the rabies viruses (RVs). RV strains varying in their genotypes and pathogenicity can be found associated with diverse reservoir species in different geographic locations (Kissi *et al,* 1995). Each RV strain is associated with a particular natural host species, usually canines and other small carnivores, such as raccoons and skunks as well as bats (Baer *et al*, 1990). RV strains associated with dogs are the major cause of human rabies worldwide due to prevalence of the virus in developing countries, whereas an RV strain associated with silver-haired bat (SHBRV) is responsible for most of the endogenous human rabies cases in the United States and Canada (Messenger *et al*, 2003).

Although rabies is generally fatal once the early clinical signs of the disease appear, prompt treatment measures that include wound cleansing and passive administration of RV-neutralizing antibodies together with active immunization can prevent development of the disease in an individual exposed to the virus (Fu, 1997). This approach, called post-exposure prophylaxis (PEP), fails to protect an infected individual once neurological signs are apparent (Centers for Disease Control and Prevention

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[CDC], 1999). Because neurological signs are indicative of virus replication in the central nervous system (CNS) tissues, it is generally considered to be impossible to clear the virus and save a RV-infected individual once RV has spread to the brain from its peripheral site of entry. However, this is not always the case. In a well-documented recent example, a rabies patient who developed evidence of antiviral immunity recovered despite developing severe neurological signs (Willoughby *et al*, 2005). There is also historical evidence of individuals surviving likely infections with the virus (Gremliza, 1953; Hattwick *et al*, 1972) as well as laboratory evidence that RV can be cleared from the CNS tissues if appropriate immune mechanisms develop (Jackson *et al*, 1989). We have recently elucidated several of the processes required to clear the attenuated RV strain Challenge Virus Standard (CVS)-F3 from the CNS (Phares *et al*, 2006). These include the development of an innate immune response in the CNS tissues and adaptive RV-specific immunity in the peripheral lymphoid organs, as well as the infiltration of immune effectors across the blood-brain barrier (BBB) (Phares *et al*, 2006). The latter is associated with increased BBB permeability to fluid phase markers primarily in the cerebellum of the infected mice (Phares *et al*, 2006). We have determined that the BBB remains intact and immune cells do not enter the CNS tissues in animals infected with SHBRV (Roy *et al*, 2007). Consequently, the virus is not cleared and SHBRV-infected animals die of rabies (Roy *et al*, 2007). Increasing BBB permeability in SHBRV-infected animals through the induction of an autoimmune CNS inflammatory response facilitates immune cell entry into the infected CNS tissues and promotes virus clearance, thereby preventing the lethal outcome of the infection (Roy and Hooper, 2007). White blood cells and virus-specific antibodies were also found to accumulate in the cerebrospinal fluid (CSF) in a patient who survived clinical rabies (Willoughby *et al*, 2005).

Unlike classical rabies, which results from the bite of an infected dog, an incident causing infection with SHBRV often goes unnoticed (Jackson and Fenton, 2001; CDC, 1999; CDC, 2007), leading to speculation that these variants may have unique attributes, possibly including the capacity to evade immune clearance. Alternatively, all pathogenic RV strains may interfere with antiviral immune mechanisms at some stage of the infection, an attribute that would explain the failure of PEP later in the disease. To distinguish between these alternatives, we have assessed BBB function and immune cell invasion into the CNS tissues of mice infected with a variety of RV strains.

Results

The outcomes of infection with different RV strains differ

Ten different RV strains ERA, PM, CVS-F3, DRV-4, Thai-DRV, CVS-N2c, CosRV, SkunkRV, SHBRV and HEP were used in the current study (Table 1). Groups of 129/SvEv mice were infected via the intradermal (ID) route with each of the RV strains and 8 days following infection, reverse transcriptase–polymerase chain reaction (RT-PCR) was used to detect the presence of viral nucleoprotein mRNA in the CNS tissues. Viral nucleoprotein message was found in the cortices and the cerebella of all infected mice, with the exception of those that had received the HEP strain (Figure 1). We were unable to detect nucleoprotein message for HEP by RT-PCR in any part of the CNS tissues (olfactory bulbs, cerebral cortex, cerebellum, brain stem, and spinal cord) following either ID or intranasal administration of HEP (data not shown). However, HEP nucleoprotein mRNA can be detected in cell culture by RT-PCR using the same set of RV-specific primers used in this study.

Table 1 Different strains of RV used for infection

RV strains	Species of origin	Passage in the laboratory	Amounts of virus in the inoculum (f.f.u)[a]	References
CVS-F3 (challenge virus standard, escape mutant)	Dog	Cell culture	1×10^5	Dietzschold *et al,* 1983
ERA (Evelyn Rokitnicki Abelseth)	Dog	Cell culture	1×10^5	Abelseth, 1964
PM (Pittman Moore)	Dog	Cell culture	1×10^5	Rupprecht *et al*, 1992
HEP (high egg passage—flurry)	Dog	Cell culture	1×10^5	Takayama-Ito *et al*, 2006
SHBRV (silver haired bat-associated rabies virus)	Bat	Neonatal mouse brain	1×10^4	Dietzschold *et al*, 2000
DRV-4 (dog rabies virus)	Dog	Neonatal mouse brain	1×10^3	Dietzschold *et al*, 2000
Thai-DRV (Thai- dog rabies virus)	Dog	Neonatal mouse brain	1×10^5	Dietzschold *et al*, 2000
CVS-N2c (challenge virus standard)	Dog	Neonatal mouse brain[b]	1×10^5	Morimoto *et al*, 1999
CosRV (Coyote rabies virus)	Coyote	Neonatal mouse brain	1×10^4	Dietzschold *et al*, 2000
Skunk RV	Skunk	Neonatal mouse brain	1×10^4	Rupprecht *et al*, 1987

[a]The number of foci developed in 20 h of *in vitro* cultured monolayer of baby hamster kidney (BHK) cells was counted as a measure of live virus particles in the inoculum.

[b]CVS-N2c was cloned in cell culture from parental CVS-24 strain, and then continued to passage in neonatal mouse brain.

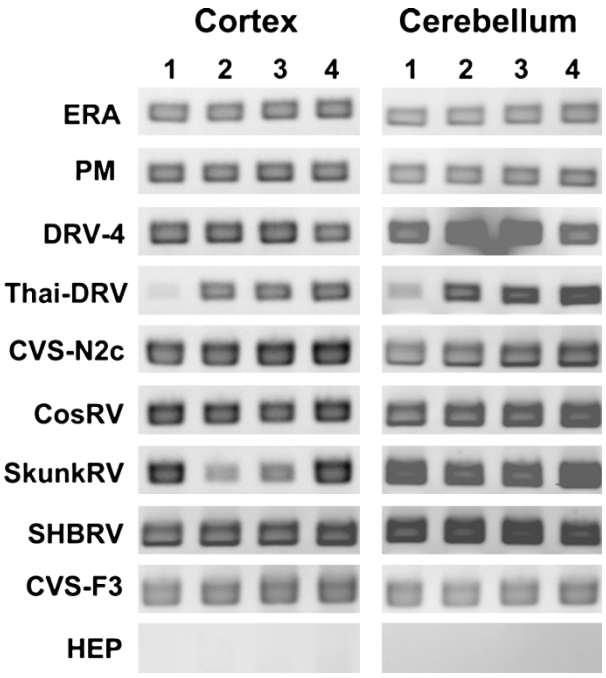

Figure 1 Detection of nucleoprotein message in the CNS of mice infected with different RV strains. RV nucleoprotein mRNA expression was detected in the CNS tissues (cerebral cortex and cerebellum) of mice infected with different RV strains 8 days previously, by RT-PCR as described in Materials and Methods. Results of four randomly chosen mice (numbered 1, 2, 3, 4) from each group of 10 mice are shown.

Similar groups of infected 129/SvEv mice were monitored for mortality. Mice infected with Thai-DRV, CVS-N2c, and SHBRV died around 8 to 12 days post infection, whereas mice infected with DRV-4, CosRV, and SkunkRV died within 12 to 17 days after infection (Figure 2). Despite the fact that the relatively less pathogenic RV strains, namely ERA, PM, and CVS-F3, reached the CNS, mice

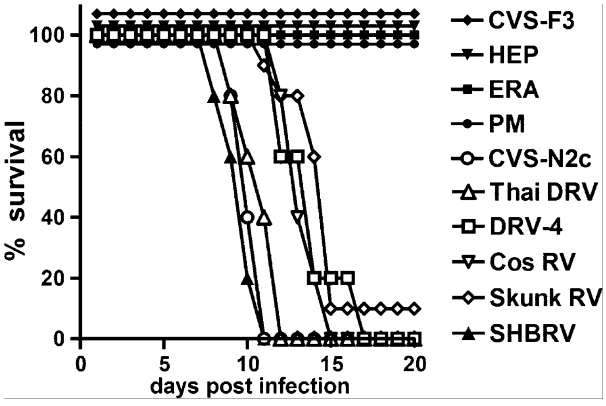

Figure 2 Survival of mice following infection with different strains of RV. Groups of 10 129/SvEv mice were infected with various RV strains listed in Table 1, via intra dermal injection as described in Materials and Methods. Mice were monitored for morbidity and mortality for 30 days. First 20 days observation is shown in the graph, there were no more deaths in any of the groups between 20 to 30 days post infection.

infected with these strains of RV survived the infection (Figure 2). Mice receiving the HEP RV strain survived without any sign of disease over a 30-day observation period.

Innate immunity is induced in the CNS by all neuroinvasive RV strains

When RV reaches the CNS tissues, it induces the expression of proinflammatory cytokines of the innate immune response (Phares *et al*, 2006; Marquette *et al*, 1996). A difference in the capacity of different RV strains to induce a CNS innate immunity has been suggested to be associated with the pathogenicity of that virus (Wang *et al*, 2005). Infection with all of the RV strains that reached the CNS of the 129/SvEv mice up-regulates the expression of genes specific for the proinflammatory cytokines tumor necrosis factor (TNF)-α and interleukin (IL)-6 (Figure 3). Moreover, the expression of these genes is elevated to more or less equivalent extents irrespective of the pathogenicity of the RV strain. Enhanced TNF-α and IL-6 expression was not seen in the CNS of mice receiving HEP RV, providing support for the likelihood that this particular RV strain is not neuroinvasive.

All RV infections induce strong RV antigen–specific antibody response

As shown in Figure 4, regardless of the pathogenicity of the infecting virus, all RV-infected animals, including those receiving HEP, developed an RV-specific immunoglobulin G (IgG) response. The serum antibody titers raised by infection with the different viruses were approximately the same, with the exception of mice infected with the ERA strain where substantially higher serum antibody titers were seen. Notably, although no evidence of either virus replication or induction of proinflammatory genes in the CNS of HEP infected mice was detected, serum RV-specific antibody titers in these mice were equivalent to those of mice infected with other RV strains.

Virus is cleared and animals survive only where anti-RV adaptive immune effectors reach the CNS

Clearance of RV from the CNS requires the invasion of the CNS tissues by immune cells, a process that primarily occurs in the cerebellum and, for CVS-F3, involves the activity of CD4 T cells and B cells (Phares *et al*, 2006, 2007). In the absence of the accumulation of these immune effectors in CNS tissues, RV infection is lethal (Roy *et al*, 2007). Eight days following infection, the expression of the mRNAs specific for CD4, CD19 (phenotypic markers for T and B cells, respectively), and κ-light chain (an indicator of antibody production by B cells) is greatly elevated in the cerebella of mice infected with the less pathogenic ERA, PM, and CVS-F3 strains of RV (Figure 5). On the other hand, no significant increase in the expression levels of these

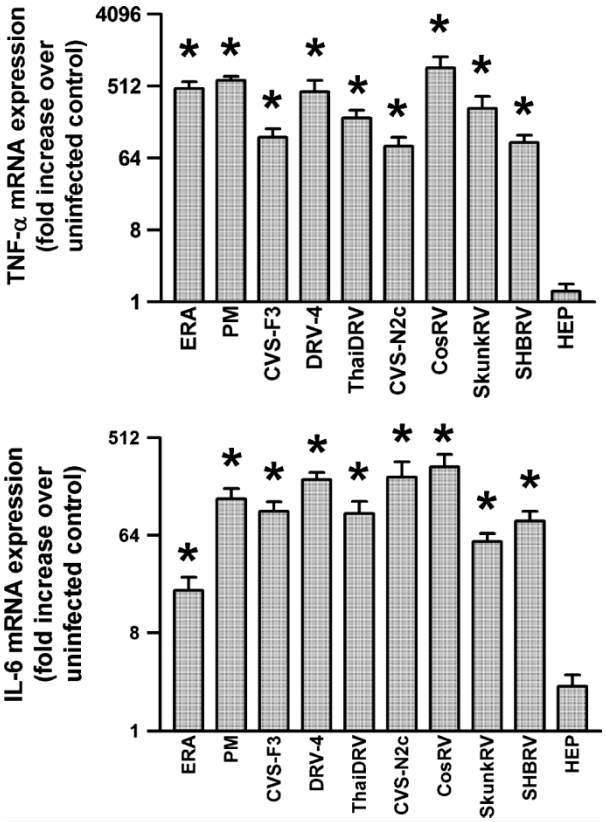

Figure 3 Innate immune response in the cerebellar tissues following infection with different RV strains. Mice were infected with different RV strains as described in Materials and Methods. Eight days after infection, cerebellar tissues were collected and levels of mRNAs specific for TNF-α and IL-6 genes were measured using QRT-PCR as described in Materials and Methods. The levels of mRNA in each sample were normalized to the level of L13 gene expression in those samples and are expressed as the fold-increase over the expression levels detected in samples from uninfected control animals. Statistically significant differences in gene expression between infected and uninfected mice were calculated using Mann-Whitney test and are denoted by the symbol * ($P < .01$; $n = 10$ in each group).

genes is seen in the cerebella of mice infected with the pathogenic RV strains DRV-4, Thai-DRV, CVS-N2c, CosRV, SHBRV, and SkunkRV (Figure 5).

As immune-cell infiltration into the CNS tissues of CVS-F3–infected mice is accompanied by an increase in BBB permeability to the fluid phase marker Na-fluorescein, we next assessed BBB integrity during infection with the different RV strains. The results paralleled the difference in immune cell invasion of CNS tissues between less pathogenic and pathogenic strains of RV. BBB permeability in the cerebellum was indeed significantly enhanced in animals infected with ERA, PM, and CVS-F3 strains, but not in animals infected with DRV-4, Thai-DRV, CVS-N2c, CosRV, SHBRV, and SkunkRV strains (Figure 6).

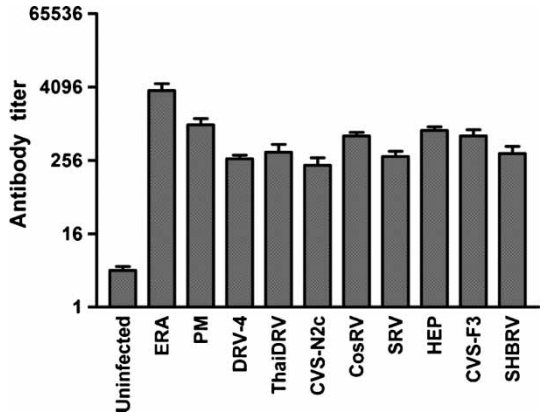

Figure 4 Serum anti-RV antibody titers of the mice infected with different RV strains. Sera were collected from the uninfected and infected mice ($n = 10$ per group) described in the legend of Figure 3 and the amounts of RV-specific IgG molecules were measured using ELISA as described in Materials and Methods. Antibody titer in the *y*-axis is the inverse of the dilution of a serum sample corresponding to the half of the maximum absorbance detected in that sample. Statistically significant (*t* test comparison with nonimmune sera) levels of RV-specific antibody titers are seen in the sera of all infected mice.

PLSJL mice are more resistant to the death from infection with different pathogenic RV strains

We have previously shown that PLSJL mice are somewhat more resistant to lethal infection with SHBRV than 129/SvEv mice (Roy and Hooper, 2007). To test if PLSJL mice are less susceptible to lethal infection with other pathogenic RV strains, groups of 129/SvEv and PLSJL mice were infected with different RV strains and monitored for clinical signs of rabies and mortality for a period of 30 days post infection. Between 8 to 15 days after infection, 129/SvEv mice infected with all RV strains, except HEP, showed prominent neurological signs of rabies (Table 2). Those infected with the pathogenic DRV-4, Thai-DRV, CVS-N2c, CosRV, SHBRV, and SkunkRV strains died within 2 to 5 days of the appearance of clinical signs, whereas 129/SvEv mice infected with the less pathogenic ERA, PM, and CVS-F3 strains survived. ERA-infected 129/SvEv mice had persistent neurological signs for over 10 to 15 days after first appearance, but nevertheless survived the infection. On the other hand, almost all of the PLSJL mice infected with ERA, PM, and CVS-F3 as well as DRV-4, CosRV, and SkunkRV survived the infection without any sign of disease (Table 2). Only one mouse in each of the CosRV- and Skunk RV–infected groups developed neurological signs and both died of rabies. However, the majority of PLSJL mice infected with Thai-DRV, CVS-N2c, and SHBRV died of rabies after developing neurological signs similar to those appearing in 129/SvEv mice infected with the same RV strains. The PLSJL mice that survived infection with these viruses did not develop any signs of clinical rabies (Table 2). As an

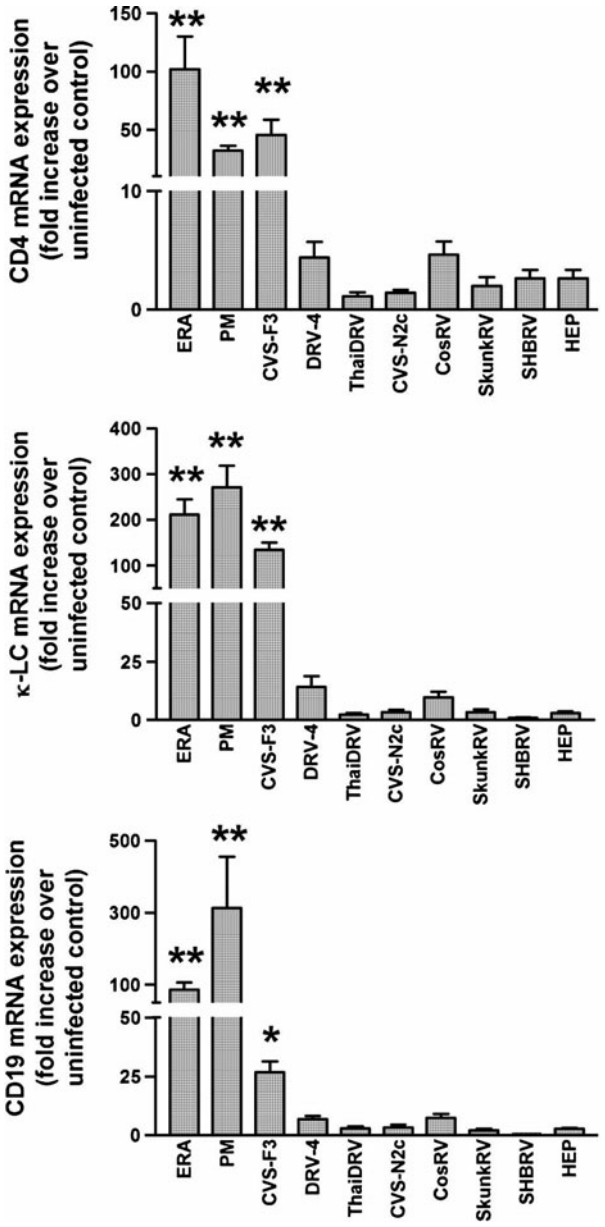

Figure 5 Accumulation of immune cells in the cerebellar tissues following infection with different RV strains. Levels of mRNAs specific for CD4, κ-light chain (κ-LC) and CD19 genes were quantified using QRT-PCR in the total RNA isolated from the cerebellum of uninfected and infected mice described in the legend of Figure 3 and normalized to L13 gene expression in those samples as described in Materials and Methods. Levels of gene expression are represented as the fold-increase in mRNA copy numbers over normalized copies in samples from uninfected control animals as described in Materials and Methods. Statistically significant differences in gene expression between infected and uninfected mice were calculated using Mann-Whitney test and are denoted by the symbol $^{**}P < .01$ ($n = 10$).

initial probe into why PLSJL mice may survive infections with RV strains that are lethal for 129/SvEv mice, we used quantitative RT-PCR to assess the amounts of virus nucleoprotein message in the CNS of mice infected with two RV strains, CosRV,

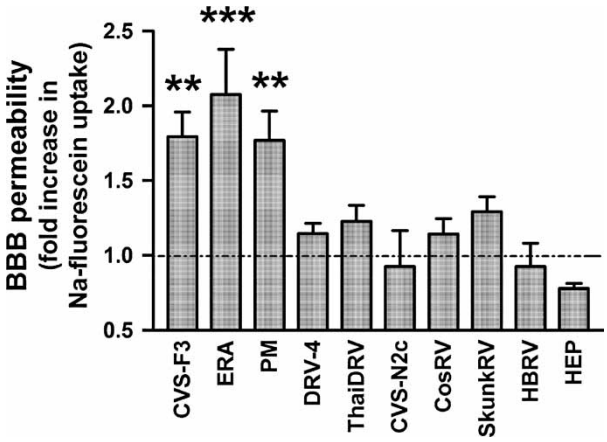

Figure 6 BBB permeability changes in the cerebellum of the mice infected with different RV strains. BBB permeability was assessed by measuring the leakage of Na-fluorescein from the circulation into the cerebellum 8 days after RV infection and are expressed as the fold-increase in Na-fluorescein uptake by the CNS tissues of RV-infected mice over that of uninfected mice, as described in Materials and Methods. The dotted line represents the BBB permeability in uninfected mice. Statistical significance of the differences in permeability between infected and uninfected mice was calculated using Mann-Whitney test and are denoted by the symbol $^{**}p < 0.01$, *** $p < 0.001$ ($n = 10$).

which is pathogenic in 129/SvEv mice but non-pathogenic in PLSJL mice, and CVS-N2c, which is pathogenic in both 129/SvEv and PLSJL mice. At 8 days of infection, the levels of nucleoprotein mRNA of the more pathogenic CVS-N2c strain were several thousands fold higher in the CNS of both 129/SvEv and PLSJL mice than those of the CosRV strain (Figure 7). However, the nucleoprotein mRNA levels of both viruses were significantly lower in PLSJL mice (Figure 7). Significantly, the only group of mice that survive, PLSJL mice infected with CosRV, exhibits the lowest viral nucleoprotein mRNA levels.

Discussion

We have previously shown that although RV-specific immunity develops in the periphery of mice infected with either the attenuated variant CVS-F3 or the highly pathogenic SHBRV, immune effectors are not delivered across the BBB into the CNS tissues of the latter (Roy *et al*, 2007). These findings led us to speculate that the mechanisms providing RV-specific immune effectors access to CNS tissues fail during infection with SHBRV and possibly other neurotropic viruses (Roy *et al*, 2007). In this study we have tested the hypothesis with respect to pathogenic RV by comparing the development of peripheral and CNS immunity in mice infected with a variety of RV strains that differ in pathogenicity. The data clearly show that a reduction in the capacity to deliver immune effectors across the

Table 2 Survival of mice following infection with different RV strains[a]

Virus strains	129/SvEv			PLSJL		
	Mortality[b]	Clinical signs	% Survival	Mortality	Clinical signs	% Survival
CVS-F3	0/20	Piloerection, weight loss	100	0/10	None	100
ERA	1/15	Piloerection, weight loss, hunched back, ataxia[c]	93	0/20		100
PM	0/15		100	0/10		100
DRV-4	10/10		0	0/10		100
SHBRV	20/20	Piloerection, weight loss, sudden onsets of severe agitation, vellication, ataxia, apnea	0	12/20	**Dying:** piloerection, weight loss, hunched back, apnea, vellication **Surviving:** no clinical sign	40
Thai-DRV	15/15		0	15/17		11
CVS-N2c	20/20		0	18/20		10
CosRV	10/10	**Dying:** weight loss, ataxia, piloerection **Surviving:** none	0	1/20	**Dying:** piloerection, weight loss, ataxia **Surviving:** none	95
Skunk RV	9/10		10	1/10		90
HEP	0/15	None	100	0/10	None	100

[a]Two strains of mice were infected with different RV strains as described in the Materials and Methods and observed for clinical signs and mortality for a period of 30 days.
[b]Number of mice that died over total number of mice used to monitor survival following an infection.
[c]Clinical signs in 129/SvEv mice infected with ERA and PM begin to show 8 to 10 days post infection and stay for 8 to 10 more days. Mice infected with DRV-4 show similar signs but die within 3 to 4 days of the first appearance of clinical rabies.

BBB into CNS tissues is common to infection with different pathogenic RV isolates. In our survey of infection with diverse RV variants, we identified only a single strain, HEP, that does not appear to spread to the CNS of 129/SvEv mice. We failed to detect viral message or evidence of innate immunity to the virus in CNS tissues from mice infected with HEP, which is considered to be poorly neuroinvasive due to the presence of glutamine at position 333 in its glycoprotein (Dietzschold *et al*, 1983; Takayama-Ito *et al*, 2006). Nevertheless, the administration of HEP stimulated a strong RV-specific antibody response. In contrast, all of the other RV strains tested were neuroinvasive, as nucleoprotein mRNAs were detectable in the CNS tissues, as were elevations in markers of innate immunity. DRV-4, Thai-DRV, CVS-N2c, CosRV, and Skunk RV were lethal for 129/SvEv mice, while similar mice infected with CVS-F3, ERA, and PM strains survived. Unlike CVS-F3–infected mice where neurological disease was not seen prior to recovery, 129/SvEv mice infected with ERA and PM exhibited overt neurological signs resembling those of mice lethally infected with DRV-4. On the basis of these observations, we can classify RV into three broad groups: (1) attenuated viruses that are poorly neuroinvasive (HEP) and a CNS-targeted immune response and changes in BBB permeability are not required for clearance; (2) attenuated neuroinvasive viruses that reach the CNS (ERA, PM, CVS-F3) but are cleared by immune effectors that cross the BBB; and (3) neuroinvasive, lethal RV where there is no evidence of BBB permeability changes and negligible invasion of immune effectors into CNS tissues (DRV-4, SHBRV, Thai-DRV, CVS-N2c, CosRV, Skunk RV).

Adoptive transfer of lymphocytes from 129/SvEv mice lethally infected with SHBRV can clear CVS-F3 from immunodeficient recipients, whereas neither these cells nor similar cells from CVS-F3–infected donors can clear SHBRV (Roy *et al*, 2007). We have interpreted these experiments as indicating that some aspect of the SHBRV infection restricts immune effectors from invading CNS tissues (Roy *et al*, 2007). PLSJL mice, which exhibit a stronger CNS inflammatory response, were found to have an elevated capacity to clear SHBRV (Roy and Hooper, 2007). To provide additional insight into whether or not some aspect of the host response is likely to be a contributing factor in RV infection, we compared the outcome of infection with various RV strains in PLSJL and 129/SvEv mice. As previously shown for SHBRV (Roy and Hooper, 2007), DRV-4, CosRV, and Skunk RV proved to be less pathogenic in PLSJL mice. In contrast, CVS-N2c and Thai-DRV are highly lethal for both PLSJL and 129/SvEv mice. The capacity of the virus to replicate and spread likely contributes to the outcome. At 8 days post infection, high levels of CVS-N2c nucleoprotein mRNAs are present in the CNS of both infected 129/SvEv and PLSJL mice, but only moderate levels of CosRV nucleoprotein mRNA in the lethally infected 129/SvEv CNS. These are somewhat reduced in the CNS of PLSJL mice that clear the infection, possibly as a consequence of the initiation of immune-mediated virus clearance. Taken together, these results indicate that both host and viral attributes contribute to

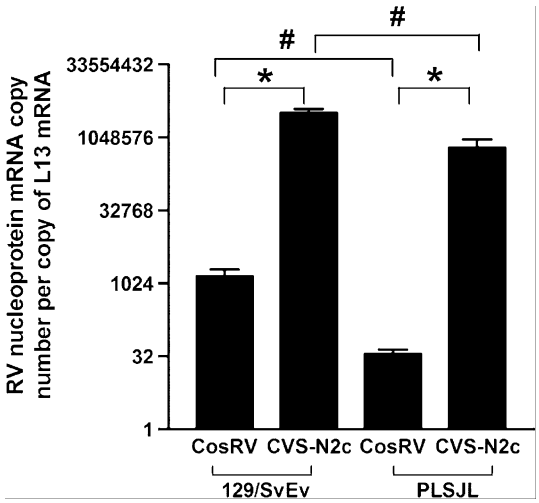

Figure 7 Nucleoprotein message levels in the cerebellar tissues of 129/SvEv and PLSJL mice infected with CosRV and CVS-N2c RV strains. Eight days post infection, QRT-PCR was used to quantify the levels of mRNAs specific for RV nucleoprotein in total RNA isolated from the cerebellar tissues of mice infected with CosRV and CVS-N2c RV strains ($n = 5$ per group). Levels normalized to L13 gene expression in the samples, as described in Materials and Methods, are presented. Significantly higher levels of nucleoprotein mRNA in CVS-N2c by comparison with CosRV-infected mice are denoted by * ($P < .05$, t test). Significantly lower levels of nucleoprotein mRNA in PLSJL mice by comparison with 129/SvEv mice are denoted by $^{\#}$($P < .05$, t test).

the lethality of RV infection. In the absence of an effective CNS immune response, RV infection is invariably lethal, as is the case for both CVS-N2c and CosRV in 129/SvEv mice. On the other hand, PLSJL mice can survive infection with CosRV but not with the more rapidly replicating CVS-N2c. Conceivably, enhancing the extent of CNS inflammation may have utility in the treatment of RV such as CVS-N2c or Thai-DRV, as has been shown for SHBRV (Roy and Hooper, 2007), but we expect that there is a level of infection at which an immune response would no longer be therapeutic.

Human rabies is almost always lethal when neurological signs are apparent in the infected host. Because neurological signs are indicative of virus replication inside the CNS tissues, it has generally been considered that once RV has reached the brain, it is impossible to clear the virus and the only hope that an individual has of surviving an RV infection is to prevent spread of the virus to the CNS. Thus people who have experienced a probable exposure to RV are treated by passive administration of preformed (exogenous) RV-neutralizing antibodies and by boosting the development of their own (endogenous) RV-specific immunity by vaccination. These treatments, together known as PEP, are thought to act by preventing the spread of RV to the CNS, as they are largely ineffective when given to people who have already manifested neurological signs of rabies (CDC, 1999). However, this is not always the case. In 2004 a patient survived clinical rabies despite developing neurological signs, evidently through the natural development of RV-specific immunity (Willoughby *et al*, 2005). The appearance of white blood cells, virus-specific antibodies, and other serum proteins in the cerebrospinal fluid of this individual indicated that the BBB had been breached and that immune effectors likely had access to infected CNS tissues (Willoughby *et al*, 2005). Our data comparing 129/SvEv and PLSJL mice suggest that host factors, in particular the capacity to deliver immune effectors across the BBB, play a large role in the ability to survive RV infection. This concept is supported by studies of rabies patients where significant serum RV-specific antibody titers are often present (Kasempimolporn *et al*, 1991), but immune-mediated changes in the CNS tissues are rare (Murphy, 1977).

Human rabies associated with dogs and other small carnivores is still a major public health problem in most parts of the world, dog rabies alone killing an estimated 50,000 to 60,000 people annually (Dressen, 1997; Jackson, 2005). In the United States, around 30,000 people receive antirabies treatment after being exposed to potentially rabid raccoons and skunks (CDC, 1999). Although there is little objective proof, PEP is generally considered to be effective unless its administration is delayed, as is often the case for lethal infections with SHBRV where exposure to the virus may not be obvious (Messenger *et al*, 2002). Proper administration of PEP has been known to fail to protect individuals bitten by rabid dogs on the face or other areas that are highly innervated, such as the fingertips (Wilde, 2007). In such cases, the virus may have reached peripheral nerves quickly after the exposure (Wilde, 2007). Our data would suggest that at some time after the virus has reached the CNS, neither PEP nor natural RV-specific immunity can protect an individual because immune effectors are unable to enter the infected CNS tissues. However, we expect that there is a window of time where immune mechanisms can clear RV from the CNS before the infection shuts down the ability to deliver immune effectors into the CNS tissues. In the current study, we found that the RV strains ERA and PM can be cleared from the brain after development of prominent neurological signs of rabies. Our RT-PCR analysis of the CNS tissues confirmed extensive virus replication in the brain during infection with these RV strains. This suggests that rabies is not invariably lethal if the virus can be cleared before neural damage, either caused by the virus or immunopathology, becomes too extensive. Thus, the predominant direct pathogenic attributes of a RV are likely related to its capacity to replicate and spread through CNS tissues (Dietzschold *et al*, 1985) as well as alter neuronal structure and function (Scott *et al*, 2008). We expect that these are in turn dependent upon the loss of the ability to support immune cell invasion across the

BBB and limit virus spread. The caveat here is that the early development of CNS immunity is likely to be protective whereas the late development may be detrimental due to immunopathology consequential to more extensive virus spread. Notably, clear evidence of immunopathology was not seen with any of the RV studied here, with the possible exception of ERA where an exaggerated immune response was evident in 129/SvEv mice: 1 out of 15 animals died whereas the rest survived with neurological abnormalities.

The concept that there is generally an inverse relationship between the pathogenicity of an RV and the capacity of the host to mediate an RV-specific CNS immune response is supported by the fact that there is little evidence of immune cell infiltration into the infected brain tissues of most individuals that succumb to rabies (Murphy, 1977). In a few human rabies cases, CNS inflammation has been reported where immunopathology may have contributed to the death (Suja *et al*, 2004). Although this has not been examined in humans, all of the RV strains studied in animal models induce strong innate, proinflammatory responses in the CNS as they reach these tissues (Marquette *et al*, 1996; Wang et al., 2006; Phares *et al*, 2006; Figure 3). Moreover, all RV are evidently highly immunogenic, with infection inducing RV-specific antibodies in the sera of infected mice (Smith *et al*, 1982; Wiktor *et al*, 1977) as well as humans (Kasempimolporn *et al*, 1991). In addition, RV can evidently be cleared by immune mechanisms without the immunopathology associated with CNS immune responses to many neurotropic viruses (Miller *et al*, 1990; Morimoto *et al*, 1996). Taken together, these observations suggest that circumventing the inability of immune effectors to cross the BBB may have therapeutic benefits in people who have developed early signs of rabies. We have previously shown in mouse models that enhancing BBB permeability by the induction of autoimmune CNS inflammation can lead to the clearance of pathogenic RV strains from the brain via naturally developed antiviral immunity. PLSJL mice, possibly due to an elevated capacity to mediate CNS inflammation, are less likely to die from rabies (Roy and Hooper, 2007). These observations not only provide insight into how a rational treatment for rabies may be developed, but also how conventional PEP may actually work. It has been well established that passive administration of rabies immunoglobulins (RIGs) without active immunization fails to protect RV-infected experimental animals (Hanlon *et al*, 2002). We speculate that active immunization accelerates the development of RV-specific adaptive immune cells capable of providing immune effectors access to infected neural tissues, a CD4 T cell–dependent process (Phares *et al*, 2007), prior to the loss of the capacity to mediate this process at the BBB. Tailoring a next generation vaccine to target a mechanism that induces functional changes in BBB integrity in a manner that is not susceptible to inhibition may provide a means to survive rabies—if immunopathology can be limited and virus-mediated neuropathology is not extensive or can be reversed.

Materials and methods

Animals and virus

129/SvEv mice were obtained from the in-house breeding colony at Thomas Jefferson University and PLSJLF1/J (PLSJL) mice were purchased from Jackson Laboratories (Bar Harbor, ME). Eight- to 10-week-old mice ($n \geq 10$ per group) were infected via intradermal (ID) route in the ear as previously described (Roy *et al*, 2007). The inoculation dose used and a brief description of each RV strain studied are presented in Table 1. The doses for the pathogenic RV strains were approximately $10 \times LD_{50}$ (ID) in mouse. Cell culture adapted strains that are not lethal for immunocompetent animals were administered at 10^5 focus-forming units, an amount that is known to cause CNS infection for CVS-F3 (Phares *et al*, 2006). All procedures were carried out according to the protocols approved by the Institutional Animal Care and Use Committee of Thomas Jefferson University.

Tissue collection

Mice were anesthetized and the thoracic cavity was surgically opened. Cardiac blood was collected and mice were transcardially perfused with 15 ml of phosphate-buffered saline (PBS) supplemented with 1 U/ml Heparin followed by 15 ml of PBS. Brains were collected and the cerebral cortex and the cerebellum were separated. Tissues were snap frozen in liquid nitrogen and stored at $-80^{\circ}C$ until further use.

RT-PCR

As an indicator of virus replication, RV nucleoprotein-specific mRNA levels were measured in the CNS tissues from infected mice by the reverse transcriptase–polymerase chain reaction (RT-PCR) technique. Briefly, total RNA was isolated from the snap frozen brain tissues of RV-infected and uninfected control mice using the Qiagen RNeasy Kit (Valencia, CA) as previously described (Phares *et al*, 2006). cDNAs were synthesized from mRNA by reverse transcription using oligo (dT) as primer. Approximately 100 ng of cDNA was subjected to PCR amplification using primers specific for a segment of nucleoprotein gene that is largely conserved among all of the virus strains used in the current study. The gene for the housekeeping ribosomal

protein L13 was quantified in each sample to ensure that the quantities of cDNA used for PCR are almost equal in all samples. The nucleotide sequences of the primers used for PCR are forward primer for RV nucleoprotein gene, 5′-TA CAATGGATGCCGA-CAAGA -3′; reverse primer for RV nucleoprotein gene, 5′-AAAGGG GCTGTCTCGAAAAT-3′; forward primer for L13, 5′-TTCCACAAGGATTGGCAGCA-3′; and reverse primer for L13, 5′-TGCTCGGATTGC-CAAAGAGT-3′. Equal amounts of the PCR products were subjected to gel electrophoresis using 1% agarose gel and bands of amplified DNA were visualized under UV light and photographed using a gel-documentation system (Bio-Rad).

Quantitative real-time PCR
The levels of mRNAs specific for tumor necrosis factor (TNF)-α, interleukin (IL)-6, CD4, CD19, and κ-light chain genes were measured in cDNA samples by quantitative real-time PCR (QRT-PCR) as previously described, using TaqMan PCR reagents (Applied Biosystems, Foster City, CA), gene-specific primers and probes, synthetic gene standards, and a Bio-Rad iCycler iQ Real Time Detection System (Hercules, CA) (Phares *et al*, 2006). In each sample, the mRNA copy numbers of a particular gene were normalized to the mRNA copy number of the housekeeping gene L13. Levels of gene expression in a test sample are presented as the fold increase over that detected in uninfected controls using the formula: n-fold increase = [mRNA copy numbers of a particular gene in a RV-infected sample/mRNA copy numbers of L13 in that sample]/average of [mRNA copy numbers of that particular gene in an uninfected mouse sample/mRNA copy numbers of L13 in that sample]. The sequences of the primers and probes used for quantitative PCR have been previously detailed (Phares *et al*, 2006).

Measurement of serum antibody titers
Levels of RV-specific total immunoglobulin-gamma (IgG) in sera from uninfected and RV-infected mice were measured by enzyme-linked immunosorbent assay (ELISA) as described previously (Roy *et al*, 2007). Briefly, serially diluted sera samples were incubated at room temperature in 96-well plates (Nalge Nunc International, Rochester, NY) coated with ultraviolet (UV)-inactivated Evelyn-Rokitnicki-Abelseth (ERA) or CVS-F3 (5 μg/ml). Captured antibodies were detected using peroxidase-conjugated anti-mouse IgG (Sigma). 3,3',5,5'-tetramethylbenzidine dihydrochloride substrate (Sigma) supplemented with hydrogen peroxide was used for color development. The reaction was terminated by the addition of 2 M H_2SO_4 to the wells and the absorbance was measured at 450 nm in a microplate spectrophotometer (Biotek, Winooski, VT). Antibody titer was calculated as the inverse of the dilution of a serum sample corresponding to the half of the maximum absorbance detected in that sample.

Assessment of BBB permeability
The extent of BBB permeability was assessed by measuring the amount of a low-molecular-weight fluorescent marker (Na-fluorescein, molecular weight 376) that leaks from the circulation into the CNS tissues as previously described (Phares *et al*, 2006). Briefly, 100 μl of 10% solution of Na-fluorescein was injected intraperitoneally and after 10 min, mice were anesthetized and transcardially perfused as described above. Snap-frozen brains were homogenized in phosphate-buffered saline (PBS) and centrifuged. Proteins from the supernatants of the tissue homogenate as well as from serum samples were precipitated with 15% trichloroacetic acid (TCA). Fluorescence in the clarified supernatant was measured at excitation and emission wavelengths of 485 and 530 nm, respectively, using a CytoFluor II fluorimeter (PerSeptive Biosystems, Framingham, MA). The amount of Na-fluorescein in the CNS tissue of each animal was normalized to the level of Na-fluorescein detected in the animal's serum using the formula: Na-fluorescein uptake = [amount of Na-fluorescein detected in the CNS tissue of a mouse/weight of the tissue]/amount of Na-fluorescein detected per microliter of serum of that mouse. Na-fluorescein uptake into the tissues of each infected animal was divided by the average uptake detected in similar tissues from uninfected control mice and the results are expressed as fold increase.

Statistical analyses
Results are expressed as the mean ± the standard error of the mean. The statistical significance of the differences in gene expression and in serum antibody levels between control and infected groups was assessed using the Mann-Whitney test, whereas the *t* test was used to test the significance of differences in virus nucleoprotein mRNA levels.

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