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Review

Murine coronavirus neuropathogenesis: determinants of virulence

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Murine coronavirus, mouse hepatitis virus (MHV), causes various diseases depending on the strain and route of inoculation. Both the JHM and A59 strains, when inoculated intracranially or intranasally, are neurovirulent. Comparison of the highly virulent JHM isolate, JHM.SD, with less virulent JHM isolates and with A59 has been used to determine the mechanisms and genes responsible for high neuropathogenicity of MHV. The focus of this review is on the contributions of viral spread, replication, and innate and adaptive immunity to MHV neuropathogenesis. JHM.SD spreads more quickly among neurons than less neurovirulent MHVs, and is able to spread in the absence of the canonical MHV receptor, CEACAM1a. The observation that JHM.SD infects more cells and expresses more antigen, but produces less infectious virus per cell than A59, implies that efficient replication is not always a correlate of high neurovirulence. This is likely due to the unstable nature of the JHM.SD spike protein (S). JHM.SD induces a generally protective innate immune response; however, the strong neutrophil response may be more pathogenic than protective. In addition, JHM.SD induces only a minimal T-cell response, whereas the strong T-cell response and the concomitant interferon-γ (IFN-γ) induced by the less neurovirulent A59 is protective. Differences in the S and nucleocapsid (N) proteins between A59 and JHM.SD contribute to JHM.SD neuropathogenicity. The hemmagglutinin-esterase (HE) protein may enhance neuropathogenicity of some MHV isolates, but is unlikely a major contributor to the high neuroviruence of JHM.SD. Further data suggest that neither the internal (I) protein nor nonstructural proteins ns4, and ns2 are significant contributors to neurovirulence. *Journal of NeuroVirology* (2010) **16,** 427–434.

Keywords: JHM and A59 strains; mouse hepatitis virus; neurovirulence, viral spread

Background

Murine coronavirus, mouse hepatitis virus (MHV), is a large, enveloped, single-stranded, positive-sense RNA virus (Figure 1). MHV can cause a wide range of illness depending on the strain and the route of infection; these include respiratory, gastrointestinal, hepatic, and central nervous system (CNS) diseases. These infections provide models for the study of encephalitis and demyelinating diseases such as multiple sclerosis (MS), hepatitis (Bender and Weiss, 2010; Weiss and Navas-Martin, 2005), and severe acute respiratory syndrome (SARS) (De Albuquerque *et al*, 2006).

Neurotropic strains of MHV cause disease in the CNS when inoculated intracranially or intranasally. Virus generally does not reach the brain of immunocompetent mice if inoculated intrahepatically or intraperitoneally. After intranasal inoculation, the virus travels transneuronally up the olfactory nerves to the olfactory bulbs where it spreads into the brain parenchyma and eventually into the spinal cord (Barnett and Perlman, 1993; Perlman *et al*, 1989, 1995; Sun and Perlman, 1995). MHV is thought to also spread through the cerebrospinal fluid, following intracranial inoculation (Wang *et al*, 1992). CNS

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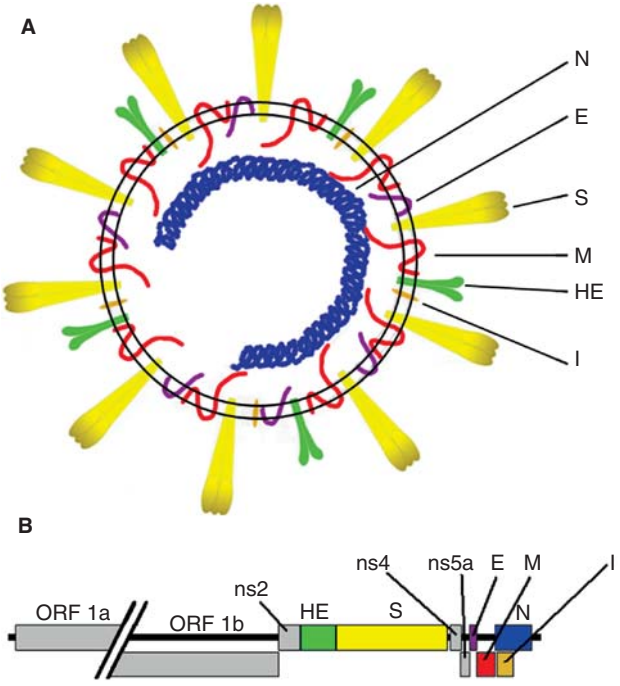

Figure 1 Schematic representation of the MHV virion and genome. (**A**) The MHV virion contains a helical nucleocapsid consisting of nucleocapsid protein (N) bound to a positive-sense RNA genome. The viral envelope contains spike peplomers (S), small envelope protein (E), and membrane protein (M). Depending on the viral strain, the viral envelope may also contain hemagglutinin-esterase protein (HE) and the internal protein (I). (**B**) MHV genome. The position of MHV genes are shown along with the relative sizes, except for ORF1a and ORF1b, which are truncated in the diagram (represented by hash marks). Note that that there will be some variation by strain.

infection leads to viremia and spread of virus to other susceptible organs such as the liver (Lavi *et al*, 1986, 1988).

Two neurotropic strains that are commonly studied are A59 and JHM. A59 is a tissue culture–adapted dual-tropic strain that infects the liver as well as the brain. A59 causes moderate to severe hepatitis and in the brain, mild encephalitis and demyelination (Lavi *et al*, 1984; Phillips *et al*, 1999); MHV-induced demyelination provides a model for multiple sclerosis. Infectious A59 is generally cleared by 10 days post intracranial inoculation, after which mice develop demyelination peaking at 3 to 4 weeks post infection (Houtman and Fleming, 1996b; Matthews *et al*, 2001; Sutherland *et al*, 1997). JHM was isolated from a paralyzed mouse (Cheever *et al*, 1949) and subsequently serially passaged in mouse brains, after which various clones were isolated. The use of multiple JHM clones, with different levels of neurovirulence, by various laboratories has necessitated the use of additional nomenclature to distinguish among these isolates. JHM.SD (formerly designated as MHV-4) (Ontiveros *et al*, 2003), the focus of this review, is among the most neurovirulent isolates (Fazakerley *et al*, 1992; Gallagher *et al*, 1990), causing lethal encephalitis. An intracranial inoculation with only a few plaque-forming units (PFU) of JHM.SD kills nearly all the infected mice within about 1 week. Like A59, JHM induces demyelinating disease in the surviving mice (Lampert *et al*, 1973; Perlman *et al*, 1987; Weiner, 1973).

Because A59 and JHM.SD display vastly different levels of neurovirulence, we have used them to investigate the viral determinants of high MHV-induced neuropathogenesis. JHM.SD has a 50% lethal dose (LD_{50}) of less than 10 PFU after intracranial inoculation of 4-week-old C57BL/6 (B6) mice, whereas A59 is approximately 1000-fold less virulent, with a LD_{50} of 3000 to 5000 PFU (Iacono *et al*, 2006; MacNamara *et al*, 2005). Both A59 and JHM.SD infect all major CNS cell types, including neurons, astrocytes, and microglia; viral antigen is found throughout the brain after infection with either virus, but JHM.SD produces more widespread infection, with larger foci of viral antigen expression (Fishman et al. 1985; Lavi *et al*, 1988; Matsubara *et al*, 1991; Parham *et al*, 1986).

Several factors contribute to enhanced lethality of highly encephalitic JHM isolates, such as JHM.SD. These include spread, replication, and adaptive and innate immunity. The kinetics of viral replication and antigen expression as well as host response are diagrammed in Figure 2. Several viral proteins have been investigated as to their role in strain-specific differences in MHV neurovirulence. A diagram of the MHV virion with structural proteins indicated as well as a schematic showing the locations of genes encoding both structural and nonstructural viral proteins is shown in Figure 1.

The expression of either the spike or nucleocapsid protein of JHM within the A59 background confers a decrease in LD_{50} to less than 10 PFU after intracranial inoculation (Cowley *et al*, 2010; Iacono *et al*, 2006; Navas and Weiss, 2003; Phillips *et al*, 1999). The virulence factors as well as the mechanisms by which viral proteins enhance neurovirulence are discussed below.

MHV spread

Neuron-to-neuron spread

In primary hippocampal neuronal cultures, JHM.SD spreads more extensively than A59, suggesting that there is an inherent difference in spread among neurons in the absence of host factors such as the immune response. In these *in vitro* cultures, both A59 and JHM.SD produce foci of infection that increase in size over time, without increasing in number; this occurs more rapidly in JHM.SD-infected cultures as compared with A59-infected cultures. JHM.SD produces very low levels of infectious virus in the medium as compared with A59 (Bender *et al*, 2010), suggesting that JHM.SD

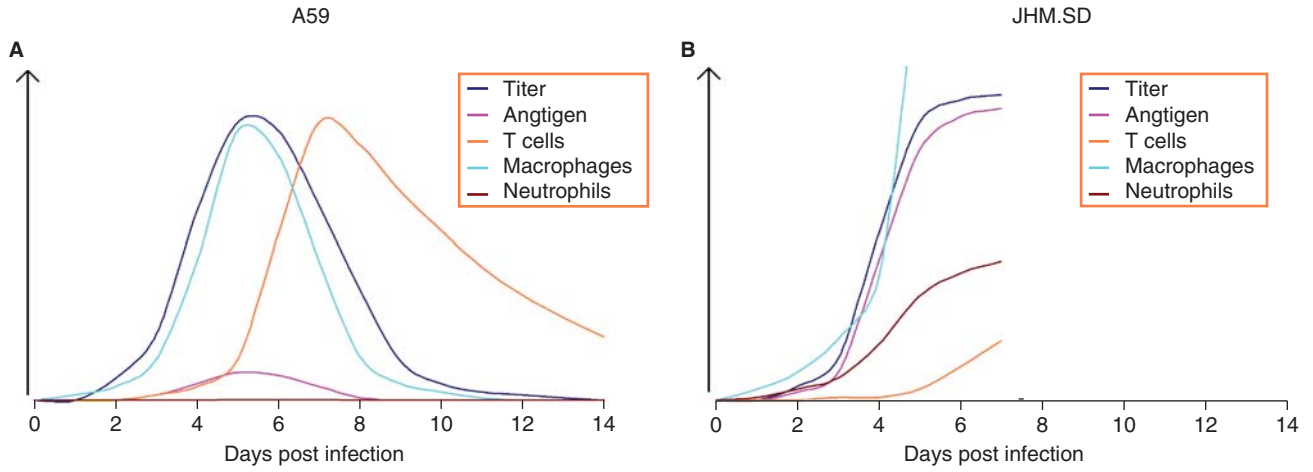

Figure 2 Diagram of parameters that correlate with neurovirulence. Shown are schematic drawings of the kinetics of replication, viral antigen, T cells, neutrophils, and macrophages in the brains of 4-week-old C57BL/6 mice infected intracranially with A59 compared with JHM.SD during the first 2 weeks of infection. Note that the graph is cropped and macrophage titers by day 7 in JHM.SD-infected mice are approximately 4 times that of A59. JHM-infected mice typically die by day 7 at a low dose (10–50 PFU) of virus.

spread is primarily neuron to neuron (Bender *et al*, 2010; Phillips *et al*, 2002). In rat hippocamapal neurons and in the neuronal cell line OBL-21, a JHM isolate used by S. Dales et al was observed to move transneuronaly in a primarily retrograde movement with some anterograde movement as well (Pasick *et al*, 1994).

The availability of reverse genetics systems has promoted the mapping of pathogenic properties to viral genes. Analysis of A59/JHM chimeric viruses demonstrated that the spike protein is largely responsible for the rapid spread of JHM. Replacement of the spike gene of A59 with that of JHM (rA59/S_{JHM}) confers increased spread in the brain and in primary hippocampal neurons, and conversely replacement of the JHM spike gene with that of A59 (rJHM/S_{A59}) results in reduced spread in the brain (Iacono *et al*, 2006; Phillips *et al*, 2002). Some JHM isolates with spike mutations are attenuated for encephalitis, but still induce demyelination. One such isolate, JHM2.2-V-1, was selected as an escape variant for neutralization by anti-spike monoclonal antibody J2.2. It has an L1114F amino acid substitution in the spike and is glial tropic; it fails to infect neurons, which most likely explains its attenuation for encephalitis (Wang *et al*, 1992). In the context of JHM/A59 chimeric viruses, the nucleocapsid protein of JHM also confers increases in the extent of viral antigen expressed in the brain; however, the mechanism is uncertain and nucleocapsid protein does not enhance neuron-to-neuron spread in primary hippocampal neuron cultures (Cowley *et al*, 2010).

Carcinoembryonic antigen–related cell adhesion molecule 1a (CEACAM1a) receptor-dependent and -independent spread

Another factor that contributes to high neurovirulence is the ability of some JHM variants, such as JHM.SD and JHM cl-2 (Taguchi *et al*, 1985), to spread in the absence of the canonical MHV receptor, CEACAM1a. This was originally demonstrated in tissue culture (Gallagher *et al*, 1992; Taguchi *et al*, 1999) and more recently in primary hippocampal neuron cultures (Bender *et al*, 2010). A very small number of cells in primary neuronal cultures derived from *ceacam1a*$^{-/-}$ mice were infected by A59 or JHM.SD; however, A59 failed to spread beyond the initially infected neurons, whereas JHM.SD spread robustly. Furthermore, when *ceacam1a*$^{-/-}$ mice were inoculated with sufficiently high titers of JHM.SD, but not A59, they developed lethal CNS disease (Miura *et al*, 2008). Thus, the ability to spread in the absence of CEACAM1a may allow JHM.SD to spread more rapidly than A59 and/or infect different neuronal subsets.

The expression of CEACAM1a protein by neurons has never been demonstrated. When measured by quantitative reverse transcriptase–polymerase chain reaction (qRT-PCR), expression was barely above background and may have been due to contamination of other, CEACAM1a-expressing, cell types (Bender *et al*, 2010). It was suggested that JHM cl-2 may first infect microglia, which had been demonstrated to express CEACAM1a (Ramakrishna *et al*, 2004), and then spread into neurons (Nakagaki and Taguchi, 2005). However, the observations that there are very few foci of infected *ceacam1a*$^{-/-}$ neurons compared to wild-type neurons and that A59 fails to spread from initially infected *ceacam1a*$^{-/-}$ neurons suggest that neurons express CEACAM1a, which is essential for spread of A59, but not JHM. It is not clear how either strain enters *ceacam1a*$^{-/-}$ neurons.

Most JHM isolates are not capable of CEACAM1a-independent spread. One such isolate, JHM.IA, like JHM.SD, is highly neurovirulent, and both viruses spread rapidly in the CNS and are uniformly fatal

in 4- to 6-week-old mice (Haspel *et al*, 1978; Knobler *et al*, 1981; MacNamara *et al*, 2005; Ontiveros *et al*, 2001; Perlman *et al*, 1987). However, JHM.SD is significantly more virulent than JHM.IA when assessed in a different model system, in which suckling mice are inoculated intranasally and nursed on dams previously immunized with JHM.IA (Ontiveros *et al*, 2003). Thus the inability of JHM.IA to carry out CEACAM1a-independent spread may contribute to subtle differences in virulence, but the fact that JHM.IA is similarly virulent to JHM.SD in adult B6 mice suggests that CEACAM1a-independent spread is likely not the only reason for enhanced virulence of JHM.SD in adult mice. Furthermore, it is quite possible that CEACAM1a-independent spread is mechanistically different in primary neurons as compared with cell lines.

Hemagglutinin-esterase

The role of hemagglutinin-esterase (HE) in neurovirulence and spread has been a subject of much speculation with evidence both for and against a role for HE in neurovirulence (LaMonica *et al*, 1991; Yokomori *et al*, 1992, 1993, 1995). However, it was demonstrated that a recombinant A59 expressing the hemagglutinin-esterase of the MHV-S strain and the spike protein of JHM.SD was more neurovirulent than an isogenic virus that does not express HE (Kazi *et al*, 2005). However, abrogation of the expression of HE within a recombinant JHM.SD genome had no affect on neurovirulence (unpublished data). This implies that HE can enhance neurovirulence for some strains, but that HE either fails to modulate JHM.SD neurovirulence or the effect of HE expression during JHM infection is not detectable due to the very high neurovirulence of JHM.

Replication

Infectious viral titers in the brains of mice infected with MHV do not always correlate with the severity of the infection. When inoculated at equivalent PFU, A59 and JHM.SD replicate to similar titers in the brain, even though JHM.SD is more lethal and expresses more intracellular antigen in the CNS (Cowley *et al*, 2010; MacNamara *et al*, 2005; Phillips *et al*, 1999). In addition, recombinant A59 expressing the JHM.SD spike gene (rA59/S_{JHM}) replicates to lower final titers than A59, even though, like JHM.SD, it has an LD_{50} of less than 10 PFU and spreads more efficiently in the CNS than A59 (Phillips *et al*, 1999). This may be due to the unstable nature of the JHM.SD spike protein, which can lead to receptor-independent conformational changes and premature inactivation (Gallagher and Buchmeier, 2001; Krueger *et al*, 2001). Consistent with this, the JHM.SD spike is more sensitive to heat and high pH treatments (Tsai *et al*, 2003). Perhaps as a consequence, JHM.SD produces more virus particles per PFU than A59 (unpublished data) and replicates to significantly 100- to 1000-fold lower titers in tissue culture (Cowley *et al*, 2010; Phillips *et al*, 1999). However, titer is indicative of disease severity in some cases, when comparing two viruses expressing the same spike protein. For example, recombinant A59 expressing the JHM nucleocapsid (rA59/N_{JHM}) is more lethal and infects more cells in the CNS than A59, and this is associated with greater replication in the brain (Cowley *et al*, 2010).

Innate immune response

Infections with A59 or JHM.SD generate different cytokine/chemokine profiles. Infections with either virus induces macrophage inhibitory factor (MIF) and tumor necrosis factor-α (TNF-α), which remain elevated throughout infection. A59 induces a strong protective interferon-γ (IFN-γ) response (Rempel *et al*, 2004a; Scott et al. 2008). JHM.SD, on the other hand, induces a weaker IFN-γ response (Rempel *et al*, 2004a; Scott *et al*, 2008) and there is one report of a stronger IFN-β response early in JHM.SD infection (Rempel *et al*, 2004a). Additionally, JHM.SD induces more macrophage chemoattractants, such as macrophage inflammatory protein-1α and -1β (MIP-1α and MIP-1β) and MIP-2, consistent with the greater number of macrophages recruited into the CNS during JHM.SD infection (Iacono *et al*, 2006; Rempel *et al*, 2004b). The robust macrophage infiltration induced by JHM.SD partially maps to the JHM.SD spike gene, as evidenced by the greater level of macrophage recruitment in the CNS of rA59/S_{JHM}-infected mice as compared with A59-infected animals (Rempel et al. 2004b; Scott *et al*, 2008).

Shortly after MHV infection, neutrophils traffic to the brain and release matrix metalloproteinases (MMPs). The combination of cytokines and MMPs causes disruption of the blood-brain barrier, which facilitates the entry of mononuclear cells (Zhou *et al*, 2002). JHM.SD infection leads to the recruitment of greater numbers of neutrophils than A59 infection, likely a result of the increased level of MIP-2 during JHM infection (Iacono *et al*, 2006; Rempel *et al*, 2004a; Scott *et al*, 2008). Depletion of neutrorophils during infection with the more attenuated DM variant of JHM resulted in a reduction in inflammatory cell infiltration, increased viral replication, and increased lethality, leading to the conclusion that neutrophils were protective against MHV infection (Zhou *et al*, 2003). However, although neutrophils are important in early control of virus, they can be pathogenic in generating toxic reactive oxygen species. Indeed, the greater recruitment of neutrophils during JHM.SD infection compared to A59 was demonstrated to be more destructive than protective (Iacono *et al*, 2006). Natural killer cells also enter the brain early in infection. They secrete IFN-γ,

which may assist in clearing virus early in infection before the adaptive immune response develops (Iacono *et al*, 2006; Rempel *et al*, 2004a).

Adaptive immune response

The adaptive immune response, both B cell and T cell, is important in restricting MHV infection. B cell–deficient mice can clear virus with normal kinetics, but in the absence of neutralizing antibodies, virus reappears in the CNS, but not the liver, about 2 weeks post infection (Lin *et al*, 1999; Matthews *et al*, 2001). SCID (severe combined immunodeficiency) and nude mice fail to clear virus, indicating that T cells are important for clearance (Fazakerley *et al*, 1992; Houtman and Fleming, 1996a). CD8 T cells are primarily responsible for viral clearance, whereas CD4 T cells are required for CD8 T-cell recruitment and maintenance (Stohlman *et al*, 1998). Adoptive transfer of CD4 T cells alone does not restrict virus replication, but depletion of CD4 T cells prevents CD8 T cell–mediated protection (Sussman *et al*, 1989). Consistent with the role for CD8 T cells in viral clearance, β_2-microglobulin–deficient mice, which are deficient in major histocompatibility complex (MHC) class I expression, are significantly more susceptible to A59 infection (Gombold *et al*, 1995).

Viral strain–dependent differences in T-cell response make significant contributions to virulence. JHM.SD induces a weak T-cell response, whereas the less virulent JHM 2.2-V-1 and A59 induce robust responses (Iacono *et al*, 2006; Marten *et al*, 2003; Rempel *et al*, 2004a). Quantification of virus specific T cells after JHM 2.2-V-1 infection showed that priming and the initiation of T-cell expansion occurs in the cervical lymph nodes (CLNs). Dendritic cells (DCs) carrying virus or viral antigen migrate from the brain to the CLNs where antigen-specific T-cell priming is believed to occur (Dorries, 2001; Stevenson *et al*, 1997); treatment of DCs with pertussis toxin prevents them from migrating from the CLNs and, in turn, prevents T-cell trafficking into the brain (Karman *et al*, 2004). After initial expansion in the CLNs, T cells expand further in the spleen before trafficking to the brain (Marten *et al*, 2003). Infectious JHM.SD is barely detectable in CLNs, whereas A59 viral titers are much higher (Macnamara *et al*, 2008). The induction of a robust T-cell response does not map to spike, as rA59/S_{JHM} induces a strong T-cell response and infectious viral titers similar to A59 are detected in CLNs (Cowley *et al*, 2010; Iacono *et al*, 2006; Macnamara *et al*, 2008; Rempel *et al*, 2004b). The cytolytic activity as well as the production of IFN-γ by CD8 T cells are crucial for the ability to clear infection from the CNS (Marten *et al*, 2001; Parra *et al*, 1999). Whereas perforin is important for clearance from astrocytes (Lin *et al*, 1997; Parra *et al*, 2001), IFN-γ facilitates cytolytic killing by up-regulating MHC expression on infected cells and mediates clearance from oligodendrocytes (Bergmann *et al*, 2003; Parra *et al*, 1999). The mechanism of viral clearance from neurons is not yet known.

Role of viral proteins in neuropathogenesis

As discussed above, spike and nucleocapsid proteins have been implicated in strain differences in virulence. Several other MHV proteins have been investigated as candidate virulence determinants. Two MHV nonstructural proteins, ns2 and ns5a, have been implicated in type I interferon antagonism (Koetzner *et al*, 2010; Zhao and Weiss, unpublished data). ns2 is predicted to have cyclic phosphodiesterase (CDP) activity. Mutations in either of two predicted catalytic histidines of ns2 of A59 confer the loss of the ability to replicate in the liver, but have no affect on neurovirulence or *in vitro* replication (Roth-Cross *et al*, 2009). Similarly, a deletion of the ns2 gene of JHM had no affect on replication *in vitro* (Schwarz *et al*, 1990), and had no detectable effect on virulence after intracranial inoculation (personal communication, J. Leibowitz and S. Perlman). These data suggest that either the ability to resist interferon signaling is not as important in the CNS as in the liver or that resistance to interferon signaling by MHV is mediated through a different mechanism in the CNS. ns5a, like ns2, is nonessential for replication *in vitro* (Yokomori and Lai, 1991), but its role in neurovirulence has not been reported.

There are data indicating that the adenosine diphosphate ribose phosphatase (ADRP; X or macro) domain of nsp3 (encoded by opening reading frame 1a [ORF1a]) (Gorbalenya *et al*, 1991; Putics *et al*, 2005, 2006), internal protein, and ns4 are not likely to be important to the neurovirulence of JHM or A59. Mutation of the predicted catalytic residue in the ADRP domain of nsp3 of A59 does not alter the ability of virus to replicate *in vitro* or the CNS (unpublished data); however, interestingly, such mutations confer reduced replication in the liver (Eriksson *et al*, 2008). Abrogation of the internal protein (I) expression in the A59 genome had no affect on replication in the CNS (Fischer *et al*, 1997) or lethality after intracranial inoculation (unpublished data), and JHM ablated for ns4 expression was similar in lethality and CNS replication as wild-type virus (Ontiveros *et al*, 2001).

Conclusion

There is still much to learn about the mechanisms responsible for the high neurovirulence of JHM.SD. These include defining the roles, if any, that the envelope proteins, membrane (M) and small membrane (E), play in neurovirulence, and determining the possible impact of the many nonstructural

proteins encoded in the replicase locus and the role of ns5a in neurovirulence. In addition, it is not fully understood how viral strain differences in the innate immune response may affect pathogenesis. It is also not well understood why JHM.SD fails to spread to and/or replicate in the CLNs, and whether this is responsible for the weak T-cell response to JHM.SD infection of the CNS and the ensuing high neurovirulence.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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