GENETIC DETERMINANTS OF VIRULENCE FOR THE A59 AND JHM STRAINS OF MOUSE HEPATITIS VIRUS

Timothy J. Cowley
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Abstract
The murine coronavirus mouse hepatitis virus (MHV) can cause a range of illnesses depending on the strain. These include hepatitis, enteritis, pneumonia, and encephalitis. Here we study the strains A59 and JHM. The A59 strain is weakly neurovirulent and causes moderate hepatitis. The JHM strain is highly neurovirulent, but is unable to replicate in the liver. Additionally, the immune response against CNS infections with JHM and A59 are different. In particular A59 induces a robust T cell response, while JHM induces a weak T cell response. We are interested in the genetic determinants of strain differences in virulence between A59 and JHM. Utilizing recombinant viruses we explored the roles of the following proteins on virulence: nucleocapsid protein (N), a multifunctional protein that when bound to RNA forms the viral nucleocapsid; internal (I) protein, a structural protein of unknown function expressed by A59, but not JHM; hemaglutininesterase protein (HE), a protein expressed by JHM, but not A59, that binds neuraminic acid and has acetylesterase activity. We demonstrate that differences in N have a major affect on neurovirulence. When the N of A59 is replaced with that of JHM the LD50 is reduced by approximately 1000 fold. This is associated with an increase in replication and greater antigen distribution in the CNS. In addition, expression of JHM N causes a minor decrease in the T cell response, but cannot account for the large differences in T cell response between A59 and JHM. Expression of A59 N by JHM did not confer hepatotropism nor did expression of JHM N by A59 block hepatotropism. N did appear to have a minor affect on hepatovirulence, as evidenced by a reduction of replication in the liver when A59 expresses JHM N. A recombinant A59 virus with a mutation in the I gene that abrogates expression had no affect on lethality of virus in the brain or liver, and a recombinant JHM virus with a mutation in the HE gene that abrogates expression had no affect on the lethality of virus in the brain after intracranial or intranasal inoculation.

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Graduate Group
Cell & Molecular Biology

First Advisor
Susan R Weiss

Keywords
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GENETIC DETERMINANTS OF VIRULENCE FOR THE A59 AND JHM STRAINS OF MOUSE HEPATITIS VIRUS

Timothy J. Cowley

A DISSERTATION

In

Cell and Molecular Biology

Presented to the Faculties of the University of Pennsylvania

in

Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

2010

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John H. Wolfe, V.M.D, Ph.D., Professor of Pathology and Medical Genetics in Pediatrics
Dedicated to my mother, father, and sister
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I thank my advisor, Susan Weiss, for providing insight and guidance. In addition to helping formulate experiments and interpret data, she has also been tremendously encouraging and supportive. I also thank the members of the Weiss lab for their support and help in troubleshooting experiments.

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I thank my family for their love and support.
ABSTRACT

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Timothy J. Cowley
Susan R. Weiss

The murine coronavirus mouse hepatitis virus (MHV) can cause a range of illnesses depending on the strain. These include hepatitis, enteritis, pneumonia, and encephalitis. Here we study the strains A59 and JHM. The A59 strain is weakly neurovirulent and causes moderate hepatitis. The JHM strain is highly neurovirulent, but is unable to replicate in the liver. Additionally, the immune response against CNS infections with JHM and A59 are different. In particular A59 induces a robust T cell response, while JHM induces a weak T cell response. We are interested in the genetic determinants of strain differences in virulence between A59 and JHM. Utilizing recombinant viruses we explored the roles of the following proteins on virulence: nucleocapsid protein (N), a multifunctional protein that when bound to RNA forms the viral nucleocapsid; internal (I) protein, a structural protein of unknown function expressed by A59, but not JHM; hemagglutinin-esterase protein (HE), a protein expressed by JHM, but not A59, that binds neuraminic acid and has acetyesterase activity. We demonstrate that differences in N have a major affect on neurovirulence. When the N of A59 is replaced with that of JHM the LD50 is reduced by approximately 1000 fold. This is associated with an increase in replication and greater antigen distribution in the CNS. In addition, expression of JHM N causes a minor decrease in the T cell response, but cannot account for the large differences in T cell response between A59 and JHM. Expression of A59 N by JHM did not confer hepatotropism nor did expression of JHM N by A59 block hepatotropism. N did appear to have a minor affect on hepatovirulence, as evidenced by a reduction of replication in the liver when A59 expresses JHM N. A recombinant A59 virus with a mutation in the I gene that abrogates expression had no affect on lethality of virus in the brain or liver, and a recombinant JHM virus with a mutation in the HE gene that abrogates expression had no affect on the lethality of virus in the brain after intracranial or intranasal inoculation.
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Chapter One

GENERAL INTRODUCTION
BACKGROUND AND SIGNIFICANCE

Coronaviridae are a family of large, enveloped, single-stranded, positive-sense RNA viruses within the order Nidovirale (58) Table 1-1. Nidovirales also include Arteriviridae, and Roniviridae, and all members share similar genome organization and replication strategies (58). "Nido" means "nest" and refers to the nested set of subgenomic mRNAs expressed by all nidoviruses (58).

Coronaviruses are named for the crown like morphology created by the spike like projections from the viral envelope which can be observed on electron micrographs (190). The Coronavirus family is divided into two subfamilies: Coronavirinae and Torovirinae. Coronavirinae are divided into there genera based on serological cross-reactivity and nucleotide sequence; these are alphacoronaviruses, betacoronaviruses, and gammacoronaviruses (previously called groups I, II, and III respectively). Betacoronaviruses are subdivided into phyloclusters A and B (previously called subgroups A and B) to emphasize that SARS-CoV of phylocluster B is more distantly related to the other betacoronaviruses (60).

<table>
<thead>
<tr>
<th>Order</th>
<th>Family</th>
<th>Subfamily</th>
<th>Genera</th>
<th>Virus</th>
</tr>
</thead>
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<tr>
<td>Nidovirale</td>
<td>Coronaviridae</td>
<td>Coronavirinae</td>
<td>Alphacoronaviruses</td>
<td>Feline infectious peritonitis virus (FIPV)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Human coronavirus-229E (HCoV-229E)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Human coronavirus-NL63 (HCoV-NL63)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Porcine transmissible gastroenteritis virus (TGEV)</td>
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<td></td>
<td></td>
<td></td>
<td>Porcine epidemic diarrhea virus (PEDV)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Betacoronavirinae</td>
<td>Phyllocluster A</td>
<td>Mouse hepatitis virus (MHV)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Human coronavirus-OC43 (HCoV-OC43)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Human coronavirus-HKU1 (HCoV-HKU1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Torovirinae</td>
<td>Phyllocluster B</td>
<td>SARS-CoV, Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Influenza bronchitis virus (IBV)</td>
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<td></td>
<td></td>
<td>Turkey coronavirus (TCoV)</td>
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<td></td>
<td></td>
<td></td>
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<td>Pheasant coronavirus</td>
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</table>

Table 1-1: Taxonomy of Coronaviruses.

Coronaviruses cause significant infections in both domestic animals and livestock. Canine coronavirus (CCoV), can cause either respiratory or enteric infection in dogs. FIPV can cause a serious systemic infection of cats that can affect multiple organs. Pigs are susceptible to transmissible gastroenteritis virus (TGEV), porcine epidemic diarrhea virus (PEDV), and hemagglutinating encephalomyelitis virus (HEV). Chickens are susceptible to infectious bronchitis
virus (IBV), and cows are susceptible to bovine coronavirus (BCoV), which causes diarrhea in calves (66). Illnesses caused by these viruses are a financial burden to farmers.

Coronaviruses also cause human illness. Most human coronaviruses cause mild respiratory illness and have been reported to be the second major cause of the common cold after rhinoviruses (113). Examples of viruses that cause mild respiratory infections are HCoV-229E (63) and HCoV-OC43 (125). Coronaviruses can also cause more severe human diseases. Severe acute respiratory syndrome coronavirus (SARS-CoV) causes severe atypical pneumonia that sometimes leads to death in otherwise healthy patients (35-36, 82, 140, 147). It was after the emergence of SARS-CoV that other human coronaviruses were identified; these included HCoV-NL63 which causes croup (16) (192) and HCoV-HKU1 which causes pneumonia (191, 207).

Murine coronavirus mouse hepatitis virus (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) disease. These infections provide models for the study of encephalitis and demyelinating diseases such as multiple sclerosis (MS), hepatitis and severe acute respiratory syndrome (SARS) (9, 26, 203). Outbreaks in rodent colonies are most commonly caused by enteric strains (67)

STRUCTURE AND LIFECYCLE OF MOUSE HEPATITIS VIRUS

Genome Organization
All coronavirus genomes consist of a long single-stranded, positive-sense RNA, of approximately 31kb in length (85, 94) Fig. 1-1). The genome has a 5’ prime cap and is polyadenylated. A highly conserved leader sequence of about 70 nt, followed by an approximately 140 nt untranslated region (5’ UTR) is located at the 5’ end of the genome; the leader sequence is also found at the 5’ end of each of the nested six to seven subgenomic mRNAs (13). The approximately 21kb
replicase locus, composed of ORF1a and ORF1b, follows the 5’ UTR. ORFS1a,b are translated into two large polypeptides, polyprotein (pp)1a, translated from ORF1a and pp1ab, translated from ORF1a and 1b via a ribosomal frameshift at the end of ORF1a (12, 57, 94). Pp1a and 1ab are proteolytically cleaved into sixteen polypeptides (nsp1-16), including an RNA dependent polymerase, helicase, several proteases, as well as other enzymatic activities and proteins with unknown functions (174). Downstream of the replicase is ORF2a, which encodes ns2, a nonstructural protein of unknown function but required for MHV replication in the liver (165). ORF2b encodes the hemagglutinin-esterase (HE) protein, an accessory structural protein, also of unknown function, expressed in some MHV strains; HE has sialic acid binding and acetyl-esterase activities (153, 162, 169, 173, 211). ORF3 encodes the spike protein, which mediates viral attachment to receptor, virus-cell fusion during entry and cell-cell fusion during viral spread (27, 48, 110). ORF4 encodes ns4, a nonstructural accessory protein that varies greatly among strains (204, 217). ORF5a encodes ns5a, a nonstructural accessory protein recently implicated as a type I interferon antagonist (79). ORF5b encodes the small envelope protein (E) which is involved in viral assembly and budding (194). ORF6 encodes the membrane protein (M) a structural protein essential to assembly (203). ORF7 encodes the nucleocapsid protein (N) which binds to RNA to form the helical nucleocapsid (203). Within the +1 reading frame of ORF7 is the internal gene, which encodes the internal (I) protein. The I protein is a small structural protein of unknown function, expressed by most, but not all, MHV strains (203). Downstream of the N gene is a 3’ untranslated region (3’-UTR) and a poly-A tail (203).
A viral particle contains a helical nucleocapsid consisting of nucleocapsid protein (N) bound to a positive-sense RNA genome. The viral envelope contains large spike peplomers (S), small envelope protein (E), and membrane protein (M). Depending on the viral strain, the viral envelope may also contain hemagglutinin-esterase (HE), and internal protein (I). Genome (B). Top shows the size and relative position of MHV genes in a representative genome (note that there will be some variation by strain). Below is a schematic representation of polyproteins 1a, and 1ab, which are encoded by the ORF1a and ORF1a/1b genes and processed into 16 nonstructural proteins (nsp)s as numbered.

Figure 1-1: Schematic representation of the MHV virion and genome. Virion (A).
Lifecycle

The MHV spike protein binds to the cellular receptor, carcinoembryonic antigen cell adhesion molecule (CEACAM)1a, a member of the Ig superfamily (205)(fig. 1-2). Some laboratory mouse strains are resistant to MHV; these strains such as SJL have the allele variant CEACAM1b instead of CEACAM1a, which also mediates viral entry when overexpressed in vitro, although very inefficiently (137). Most MHV strains enter the cytoplasm by fusion directly at the plasma membrane, although at least one MHV strain, MHV-2, enters through a cathepsin dependent endocytotic pathway (53, 80, 130, 161). After entering the cytoplasm the genomic RNA functions as an mRNA for the translation of the viral RNA-dependent RNA polymerase. The entire replication cycle occurs in the cytoplasm. Complementary (negative sense) RNA genomes and mRNA are produced by a process involving transcription of noncontiguous sequences in which the leader complement is added to the 3’end of each negative sense subgenomic transcript. The negative sense full-length and subgenomic RNAs then serve as templates for positive sense genomes and a nested set of six subgenomic mRNA. Each mRNA serves as mRNA for the 5’ gene, not contained in the next shorter mRNA (fig. 1-2). M and E proteins are targeted to the intracytoplasmic membranes and spike protein is present on the plasma membrane as well as intracellular membranes. Virus assembly occurs as viral nucleocapsids bud into the endoplasmic reticulum-Golgi intermediate complex (ERGIC) membranes forming vesicles that travel to the plasma membrane and release virus (29).
Figure 1-2: Schematic representation of the virus lifecycle. Spike binding of CEACAM1a at the plasma membrane triggers fusion and allows viral nucleocapsid to enter the cytoplasm. Genomic RNA functions as the mRNA for the synthesis of the replicase proteins. Negative sense genomic RNA and subgenomic RNAs are used as templates to produce positive sense genomic RNA and a nested set of subgenomic mRNAs. Proteins are translated and virions are assembled by budding into intracellular membranes. Virus containing vesicles travel to the cell membrane where they fuse and release virus. Abbreviations: Rough endoplasmic reticulum (RER), Endoplasmic reticulum – Golgi intermediate compartment (ERGIC)

MOUSE HEPATITIS VIRUS PATHOGENESIS

Multiple factors influence the pathogenesis of mouse hepatitis virus. Different MHV strains vary greatly in their organ tropisms and virulence. The age and strain of the infected mouse and the route of infection are also important determinant of pathogenesis.
CNS Pathogenesis – Acute Disease

Neurotropic strains of MHV, such as A59 and JHM (see Table 1-2 for list of viral strains), cause disease in the CNS when inoculated intracranially or intranasally, but virus does not reach the brain of immunocompetant mice if inoculated intrahepatically or intraperitoneally. After intranasal inoculation the virus travels transneuronally up the trigeminal and olfactory nerves to the olfactory bulb where it spreads further and eventually reaches the spinal cord (6, 149, 151, 182). Virus is thought to also spread through the cerebrospinal fluid, following intracranial inoculation (199). CNS infection with some strains leads to viremia and spread of virus to other susceptible organs such as the liver (89-90).

<table>
<thead>
<tr>
<th>Virus</th>
<th>Alternative Names</th>
<th>Relevance</th>
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<tr>
<td>JHM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>JHM.SD</td>
<td>MHV-4</td>
<td>highly neurovirulent, CEACAM-independent spread, non-hepatovirulent</td>
</tr>
<tr>
<td>JHM.IA</td>
<td></td>
<td>highly neurovirulent, no CEACAM-independent spread, non-hepatovirulent</td>
</tr>
<tr>
<td>JHM.V-1</td>
<td>JHM 2.2V-1</td>
<td>neuroattenuated relative to JHM.SD, non-hepatovirulent, demyelinating</td>
</tr>
<tr>
<td>A59</td>
<td></td>
<td>moderately to severely hepatitic, weakly neurovirulent, demyelinating strain</td>
</tr>
<tr>
<td>MHV-2</td>
<td></td>
<td>nondemyelinating, nonencephalitic, causes meningitis and hepatitis</td>
</tr>
<tr>
<td>MHV-3</td>
<td></td>
<td>severely hepatoviral strain</td>
</tr>
<tr>
<td>rA59/Sd,jhm</td>
<td>SJHM-RA59, R13</td>
<td>A59 expressing JHM.SD spike</td>
</tr>
<tr>
<td>rJHM/Sd,a59</td>
<td>SA59-RJHM</td>
<td>JHM.SD expressing A59 spike</td>
</tr>
<tr>
<td>SMHV2-RA59</td>
<td>Penn98-1/2</td>
<td>A59 expressing MHV-2 spike</td>
</tr>
<tr>
<td>rA59/NJm</td>
<td></td>
<td>A59 expressing JHM.SD nucleocapsid protein</td>
</tr>
<tr>
<td>rA59/Sd,jhm/Njhm</td>
<td></td>
<td>A59 expressing JHM.SD spike and nucleocapsid protein</td>
</tr>
<tr>
<td>rJHM/Na59</td>
<td></td>
<td>JHM.SD expressing A59 nucleocapsid protein</td>
</tr>
<tr>
<td>rJHM/Sd,a59/Na59</td>
<td></td>
<td>JHM.SD expressing A59 spike and A59 nucleocapsid protein</td>
</tr>
<tr>
<td>rMHV-A59S-HE†</td>
<td>rMHV-HE†</td>
<td>A59 expressing MHV-S hemagglutinin-esterase (HE)</td>
</tr>
<tr>
<td>rMHV-A59S-HE*</td>
<td>rMHV-HE*</td>
<td>A59 containing a nonexpressed MHV-S HE</td>
</tr>
<tr>
<td>rMHV-A59S-HE*°</td>
<td>rMHV-HE*</td>
<td>A59 expressing an esterase activity deficient MHV-S HE</td>
</tr>
<tr>
<td>rMHV-JHMS-HE†</td>
<td></td>
<td>A59 expressing JHM.SD S and MHV-S hemagglutinin-esterase</td>
</tr>
<tr>
<td>rMHV-JHMS-HE*</td>
<td></td>
<td>A59 expressing JHM.SD S and containing a nonexpressed MHV-S HE</td>
</tr>
<tr>
<td>rMHV-JHMS-HE* °</td>
<td></td>
<td>A59 expressing JHM.SD S and an esterase activity neg. MHV-S HE</td>
</tr>
</tbody>
</table>

Table 1-2: Virus Names and Descriptions.

Two neurotropic strains that were used in this study 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 it causes mild encephalitis followed by chronic demyelination (92, 155). Infectious A59 is generally cleared by ten days post intracranial inoculation after which mice develop chronic demyelination peaking around three to four weeks post infection (69, 123, 184). JHM, a highly neurovirulent strain, was isolated from a paralyzed mouse (14) and
subsequently serially passaged in mouse brains, after which various clones were isolated. JHM.SD (MHV-4)(138) is among the most neurovirulent isolates (44, 54) and causes lethal encephalitis. An intracranial inoculation with only a few PFUs of JHM.SD kills nearly all the infected mice within about one week. Like A59, JHM induces demyelinating disease in the surviving mice (88, 150, 202).

Our lab uses the A59 and JHM.SD strains, which display vastly different levels of neurovirulence, to investigate the viral determinants of high MHV induced neuropathogenesis. JHM.SD has a fifty percent lethal dose (LD$_{50}$) of less than 10 PFU after intracranial inoculation of 4 week old C57BL/6 (B6) mice, while the LD$_{50}$ of A59 is approximately 3,000-5,000 PFU, approximately 1000 fold higher (72, 112). A59 and JHM.SD infect all major CNS cell types; viral antigen is found in the olfactory bulb, basal ganglia, subiculum, hypothalamus, and hindbrain, but JHM.SD produces larger foci of infection and appears to be more prevalent in the hippocampus (47, 89, 119, 141). The more extensive spread of JHM.SD is thought to contribute to the differences in virulence. A59 and JHM.SD might be different in tropism for certain neuronal subsets. The innate and adaptive immune responses against A59 and JHM.SD are different, and in particular a more robust T cell response elicited by A59 is thought to contribute to viral clearance and host survival.

The differences in neuronal spread are thought to be responsible, at least in part, for the differences in neurovirulence between A59 and JHM.SD. In primary hippocampal neuronal cultures both A59 and JHM.SD produce foci of infection that increase in size over time, which occurs more rapidly in JHM.SD infected cultures as compared with A59. The number of foci did not increase with time, suggesting that spread is primarily transneuronal (8, 156). In addition, JHM.SD produced very low levels of infectious virus in the medium as compared with A59 (8). In rat hippocampal 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 (145).
Another factor thought to contribute to neurovirulence is the ability of JHM.SD, but not A59, to spread in the absence of CEACAM1a. This ability was originally demonstrated in tissue culture (52) and more recently in primary hippocampal neuron cultures (8). A very small percentage of cells in primary hippocampal neuron cultures derived from CEACAM1a−/− mice were able to be infected by both A59 and JHM.SD; however, A59 failed to spread beyond the initially infected neurons, while 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 (127), implying 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-PCR (qRT-PCR), expression was barely above background and may have been due to contamination of other, CEACAM1a expressing, cell types (8). It was suggested that JHM cl-2, another JHM variant capable of CEACAM1a independent spread (186), may first infect microglia, which had been demonstrated to express CEACAM1a, and then spread into neurons (128). However, the observations that there are only a 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 do express CEACAM1a. That is, these data suggest that CEACAM1a expression mediates initial infection of A59 and JHM.SD and is essential for spread of A59. These data suggest that the low expression of CEACAM1a by neurons may be limiting for the rate of spread of A59, while JHM.SD is able to spread more rapidly because it is not dependent on CEACAM1a expression.

Most JHM isolates are incapable of CEACAM1a independent spread. JHM.IA is one such variant. Like JHM.SD, JHM.IA is highly neurovirulent, and both viruses spread rapidly in the CNS and are uniformly fatal in four to six week old B6 mice after intracranial infection of only a few PFU (65, 77, 112, 139, 150). However, JHM.SD is significantly more virulent in a model in which suckling
mice are inoculated intranasally and nursed on dams previously immunized with JHM.IA (138). Thus the inability of JHM.IA to engage in CEACAM1a independent spread may contribute to subtle differences in virulence, but the fact that JHM.IA is similar to JHM.SD in virulence in adult B6 mice suggests that CEACAM1a independent spread is likely not the primary reason for enhanced virulence of JHM.SD compared to A59.

**CNS Pathogenesis – Chronic Disease**

Mice that survive acute CNS infection with A59 and neuroattenuated strains of JHM develop chronic demyelinating disease after clearing infectious virus, and this is dependent on spread into the spinal cord. Spread through blood, cerebral spinal fluid (199), and transneuronally (182) have all been suggested mechanisms by which virus reaches the spinal cord. Virus is first detected in gray matter (mostly neurons) of the spinal cord and later in white matter (predominately in astrocytes), which suggests a transneuronal route of spread (182). Demyelination occurs in the white matter of the spinal cords of mice that have recovered from acute encephalitis and cleared infectious virus. Demyelination after A59 infection usually occurs two to three weeks post infection leading to pronounced weakness and hind limb paralysis. Mice undergo remyelination and often recover from demyelinating disease, but those that do not die from it. Lesions of demyelination are observed in spinal cords and include immune infiltrates of lymphocytes and lipid containing macrophages (88, 90, 179). Both persistence of viral RNA (91) and an anti-MHV immune response are necessary for demyelinating illness.

After clearance, viral RNA persists in the CNS. During infection with the attenuated JHM V-1 (JHM 2.2V-1), viral RNA persists and is correlated with chronic disease and paralysis (116-117). It is unclear how virus persists. Infectious virus was not recoverable from A59 infected demyelinated animals even when brains and spinal cords were isolated, homogenized and used to infect naïve animals (unpublished). However, viral genomes and mRNA are detectable by qRT-PCR and virus epitope specific T cells are present (Bender et al., mss in preparation). The presence of RNA in the spinal cord is not enough to induce demyelinating disease, but must be
present in the white matter. Evidence for this comes from infection with a virus expressing MHV-2S in the A59 background (SMHV2-RA59), which persists in gray, but not white, matter of the spinal cord and does not induce demyelination (23-24).

Demyelination is thought to be mediated by infiltrating macrophages and activated astrocytes (22, 55, 181, 209). When splenocytes are adoptively transferred to infected RAG−/− mice, macrophages and microglia traffic to the sites of demyelination (209). Astrocytes in the CNS of mice with persistent JHM RNA secrete the inflammatory molecules TNF-α, IL-β, IL-6 and iNOS (181). The secretion of these molecules might have toxic affects on oligodendrocytes that lead to demyelination. Most of the evidence suggests that MHV induced demyelination does not involved an autoimmune response against myelin itself. Autoreactive T cells and antibodies have never been described in MHV infection; however, in one study splenocytes from A59 infected mice proliferated when cultured with both viral and myelin antigens suggesting that T cells specific to myelin basic protein were present (62).

After acute infection is resolved T and B cells remain in the CNS. It is unclear how these cells are maintained, but it appears to correlate with the presence of persistent viral RNA in the spinal cord (116). Persisting CD8 T cells, in the case of JHM.V-1 infection are retained in a semi-activated state and secrete IFN-γ upon stimulation but are not cytolytic (116). The functionality of persistent CD8 T cells after A59 infection were intermediate of that of the protective memory T cells elicited by acute infections of LCMV Armstrong and the exhausted T cells from chronic infections of clone 13 (9).

Persisting T and B cells may mediate demyelination. Recombinase-activating gene deficient (RAG−/−) mice are protected from JHM.IA induced demyelination until they receive adoptive transfers of virus-specific splenocytes from immunocompetent mice (200). CD8 T cells in particular are associated with demyelination (208) and demyelination is dependent on the ability of the CD8 T cells to produce IFN-γ (208). The importance of T cells to demyelination appears to
be dependent on viral strain. A59, unlike JHM.IA, causes demyelination in RAG<sup>−/−</sup> mice (122) and A59 infected mice depleted for either CD4 or CD8 T cells still exhibited demyelination (184). There are contradictory data regarding the possible role of bystander CD8 T cells in mediating demyelination (15, 64). In experiments where bystander CD8 T cells contributed to demyelination IFN-γ secretion was required (21).

**Liver Pathogenesis**

Hepatovirulent strains of MHV can reach the liver following various routes of inoculation and cause hepatitis. Studies of hepatitis are usually carried out after intraperitoneal or intrahepatic inoculations. Virus inoculated by this route does not infect the CNS. As with CNS disease, severity of hepatitis depends on the viral strain, host strain, and host age.

The A59 isolate that our lab studies causes moderate to severe hepatitis in the liver (92, 155). A59 infects both sinusoidal lining cells, which consist of Kupffer and endothelial cells, and parenchyma cells, which consists mostly of hepatocytes (132). Viral titers peak around five days post infection, and mice that survive infection usually clear virus in about one week. Hepatitis from infection with A59 is characterized by regions of focal necrosis, mononuclear cell infiltrates, and fibrin deposition (11, 132). Another hepatovirulent strain, MHV-3, causes more severe hepatitis. This is evidenced by differences in LD<sub>50</sub>s. A59 has an LD<sub>50</sub> of about 5x10<sup>3</sup> PFU after intrahepatic inoculation, while MHV-3 is almost uniformly fatal at doses less than 10 PFU. Since MHV-3 has been more extensively studied than A59 in relation to liver pathogenesis, it will be the focus below.

MHV-3 causes severe hepatitis in susceptible mouse strains, which include DBA/2, BALB/c and C57BL/6 (93). MHV-3 causes a less virulent persistent infection in semisusceptible strains such as C3H and in F1 crosses between susceptible and resistant strains (93). In resistant strains MHV-3 replicates to high titer, but causes minimal damage and is cleared (93).
About twelve hours post infection necrotic lesions form in the livers of MHV-3 infected susceptible mice, and these are associated with infiltrates of neutrophils and mononuclear cells (31). These lesions continue to grow until they become confluent. Viral titers in the liver peak around three to four days post infection and remain high until the death of the animal, which usually occurs within one week. MHV-3 infection is generally lethal in susceptible mice of eight weeks of age or younger.

Resistance of A/J mice to MHV-3 is age dependent. Mice younger than 2 weeks or age are susceptible and consistently develop lethal hepatitis (93), while mice of 4 weeks or older are nearly uniformly resistant (93, 97). In A/J mice virus replicates to about the same peak titer as in susceptible mice (93, 98), but induces almost no pathology. Virus is generally cleared around 1 week post infection. In semisusceptible strains such as C3H, mice that survive the initial acute disease go on to develop a persistent infection (93).

Differences in infection of primary cell cultures correlates with susceptibility to virus. MHV-3 is able to replicate in both susceptible and resistant strain splenic and peritoneal macrophages, but will only form syncytia in macrophages from susceptible strains (37-38). MHV-3 replicates more slowly and to lower titers in macrophages and hepatocytes of restraint A/J mice than in cells derived from susceptible C57BL/6 mice (4, 37-38). Similarly MHV-3 replicated slower and to lower titers in resistant A/J MEFs, Kupffer cells, and endothelial cells compared to susceptible C57BL/6 MEFs and BALB/c Kupffer and endothelial cells (87, 148).

While MHV-3 replicates to high titer in both susceptible and resistant strains only the susceptible strains develop with fulminate hepatitis. MHV-3 infection in BALB/c mice, but not A/J mice, leads to fibrin deposition and the blockage of sinusoids. This results in necrotic lesions and edema.

Host strain differences in MHV induction of fibrinogen-like protein 2 (fgl2) appear to be largely responsible for differences in susceptibility. Fgl2 is produced in both a membrane associated and
soluble form. The membrane associated form has procoagulant activity (PCA) through its function as a prothrombinase (cleaves prothrombin to thrombin) and is induced in macrophages by MHV-3 and A59 (50, 136). The soluble form acts as an immunosuppressant via interaction with the Fcy Receptor IIB and suppresses T cell proliferation and DC maturation (104-105). Both forms are induced in susceptible mice and MHV-3, and the importance of the T cell response will be addressed later.

MHV-3 infection induces fgl2 in susceptible, but not resistant, mouse strains and this accelerates the formation of fibrin clots (18, 32, 97). Semisusceptible C3H mice induce intermediate levels of fgl2 (18). T cells in susceptible, but not resistant strains, induce macrophages to produce more PCA (18). Fgl2/- susceptible mice have enhanced survival and development of necrotic lesions is delayed and greatly reduced (114). In addition blocking PCA through the drug 16,16-dimethylprostaglandin E2 (1) or anti-PCA antibodies prevents the development of fulminant hepatitis (50, 100) but do not affect viral titers.

**IMMUNE RESPONSE TO MOUSE HEPATITIS VIRUS INFECTION**

**CNS Immune Response**

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 (222). If neutrophils are depleted during infection with the more attenuated DM variant of JHM there is a reduction in inflammatory cell infiltration, increased viral replication, and increased lethality (223). While neutrophils are an important to early control of virus, they can be pathogenic. Neutrophils generate toxic reactive oxygen species and the greater recruitment of neutrophils during JHM.SD infection compared to A59 may contribute to enhanced pathogenesis (72).
Infections with A59 or JHM.SD generate different cytokine profiles. Infections with either virus induce macrophage inhibitory factor (MIF) and tumor necrosis factor-α (TNF-α), which remain elevated throughout infection. A59 induces a strong protective IFN-γ response (163, 171). JHM.SD, on the other hand induces a weaker IFN-γ response (163, 171) and there is one report of a stronger IFN-β response early in JHM.SD infection (163). Additionally, JHM.SD induces more macrophage chemoattractants, such as macrophage inflammatory protein 1-α and -β (MIP-1α and MIP-1β) and MIP-2, consistent with the greater number of macrophages recruited during JHM.SD infection (72, 164).

Natural Killer cells also enter the brain early in infection. They produce IFN-γ which is thought to be crucial for the clearance of virus from the brain, and may help clear virus early in infection before the adaptive immune response takes affect (72, 163).

Both the B and T cell responses of the adaptive immune response are important to fighting 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 two weeks post infection (101, 123). SCID and nude mice fail to clear virus, suggesting that T cells are important for clearance (44, 68) CD8 T cells are primarily responsible for viral clearance while CD4 T cells are required for CD8 T cell recruitment and maintenance (178). Adoptive transfer of CD4 T cells alone do not restrict virus replication, but depletion of CD4 T cells prevent CD8 T cell mediated protection (183). Consistent with the role of CD8 T cells in viral clearance, beta-2 microglobulin deficient mice, which are deficient in MHC class I expression, are significantly more susceptible to A59 infection (56).

Viral strain dependent differences in T cell response make significant contributions to virulence. JHM.SD induces a weak T cell response, while the less virulent JHM.V-1 and A59 viruses induce robust responses (72, 117, 163). Antigen-specific T cells in the brain are thought to be primed following antigen presentation in the cervical lymph nodes (CLN) (34, 177). JHM.SD titers
are barely detectable in the CLN, while A59 viral titers are much higher (111). Quantification of virus specific T cells after JHM.V-1 infection showed that priming and the initiation of T cell expansion occurs in the CLN. Dendritic cells (DC)s, carrying virus or viral antigen migrate from the brain to the CLN and these DCs are required for T cell trafficking into the brain (74). After initial expansion in the CLN, T cells expand further in the spleen before trafficking to the brain (117). The cytolitic activity as well as the production of IFN-γ by CD8 T cells are crucial for their ability to clear infection from the CNS (115, 143). While perforin is important for clearance from astrocytes (102, 144), IFN-γ facilitates cytolytic killing by upregulating MHC expression on infected cells and is believed to aid in clearance from oligodendrocytes (10, 143). The mechanism of viral clearance from neurons is not yet known.

Liver Immune Response

T and B cells are both contribute to viral clearance from the liver. MHV-3 infection of resistant A/J mice becomes lethal if the T and B cells are depleted (39, 106). T cells are crucial to control of infection, since depletion of T cells causes a decrease of IFN-γ production, and the IFN-γ response is required for protection (106, 196-197). B cells are demonstrated to be important from experiments where passive transfer of antibody from resistant mice to susceptible mice, while not protective, prolongs survival (93, 157).

Differences in the B and T cell responses correlate with host strain differences in susceptibility. Resistant A/J mice mount a strong antibody response, semisusceptible C3H mice mount a weaker antibody response, while susceptible BALB/c mice fail to mount an antibody response (99, 195). The CD4 T cells derived from immunized BALB/c mice were predominantly Th2, while CD4 T cells from resistant A/J mice were predominately Th1 (157). Th1 T cells control infection through the production of IFN-γ and help prevent hepatitis by suppressing fgl2 production by macrophages, while the Th2 T cells from BALB/c mice fail to control infection and stimulate macrophages to produce fgl2 (17, 157). While the induction of membrane bound fgl2 contributes to hepatitis (1, 18, 32, 50, 97, 100, 114, 136), fgl2 is also produced in a soluble form that can
inhibit the T cell response by blocking DC maturation and T cell proliferation (104-105). Additionally the T and B cell response may be less effective in susceptible mice because T and B cells of susceptible, and not resistant mice, can be infected and lysed by MHV-3 (86).

There are also differences between resistant and susceptible strains in the macrophage response. A/J macrophages restrict MHV-3 replication in the presence of both type I and type II IFN, while BALB/c macrophages do not (108). IFN-γ downregulates CEACAM1a in A/J macrophages, but does not in BALB/c macrophages, and the downregulation of CEACAM1a could assist in controlling infection by making cells more resistant (193). The macrophages and lymphocytes from younger mice are not as good at synthesizing type I and type II IFN, and this may explain why younger A/J mice are not resistant to MHV-3 infection (109). Cytokine secretion also varies by host strain, and BALB/c macrophages secrete more TNF-α and IL-1 in response to MHV-3 infection (107, 158).

In C57BL/6 mice a combination of TNF-α and IFN-γ was able to cause necrotic and apoptotic lesions, fgl2 expression by endothelial cells and macrophages, and fibrin deposition in the absence of infection (105). This suggests that TNF-α expression by BALB/c macrophages could be pathogenic and that depending on the cytokine environment IFN-γ may be pathogenic as well.

MOUSE HEPATITIS VIRUS PROTEIN FUNCTIONS AND ROLES IN PATHOGENESIS

Structural Proteins

Spike Protein (S): The spike protein is a 180kDa type I transmembrane glycoprotein that forms 20 nm protrusions from the viral envelope (25, 48, 110). Spike forms homotrimers (9) and is responsible for both attachment to the MHV receptor, CEACAM1a, and fusion of the virion with the cell membrane (48, 110). Infected cells express spike and the surface allowing for syncytia formation (27). Spike protein is cleaved by a furin-like protease into two 90kDa non-covalently
associated polypeptides, S1 and S2 (48, 110). S1, the N-terminal polypeptide, forms a globular head domain that serves as the receptor binding domain (RBD) and contains a hypervariable region (HVR) (48, 110). S2, the C-terminal polypeptide, has the fusion peptide and two heptad repeats (HR1 and HR2) believed to form coiled-coil interactions involved in membrane fusion (48, 110).

Spike is a major determinant of tropism. For example, exchanging the MHV spike for that of feline infectious peritonitis virus is enough to alter the species specific tropism from murine to feline cells in tissue culture (83). Though it is a major determinant of tropism spike is not the sole tropism determinant. A chimeric virus expressing the JHM.SD spike within the A59 background was able to infect the liver and induce hepatitis at high doses despite the fact that JHM.SD fails to replicate in the liver. In addition, exchanging the JHM.SD spike for that of A59, did not allow the virus to replicate in the liver, even at high doses (133). Also a chimeric virus expressing MHV-2 spike in A59 background caused encephalitis, even though MHV-2 is not encephalitic (23).

Spike is also major determinant of neurovirulence. A recombinant A59 virus in which the spike was replaced with that of JHM.SD (rA59/S_{JHM}) exhibits a lower LD_{50} than wild type recombinant A59, and a recombinant JHM virus in which the spike was replaced with that of A59 (rJHM/S_{A59}), had a higher LD_{50} than wild type recombinant JHM.SD (72, 133, 155). Exchanging spikes also altered the extent of viral spread in the CNS; rA59/S_{JHM} spread more extensively than rA59 and rJHM/S_{A59} spread less extensively than JHM.SD (72, 156). It was also determined that rA59/S_{JHM} infections of the CNS induce the production of greater levels of macrophage chemoattractants and lead to the recruitment of more macrophages into the brain than infections with rA59, suggesting that differences in spike are largely responsible for differences in macrophage recruitment (164, 171).

There are multiple isolates of the JHM strain that differ in virulence, and these differences have been largely mapped to differences in the spike gene. For example, JHM.SD is more
neurovirulent than JHM.IA in a suckling mouse model, where the dam is vaccinated with JHM.IA and the suckling mice are infected intranasally. JHM.SD is capable of spreading among cells in culture in the absence of the CEACAM1a receptor, while JHM.IA is not, and this has been assumed to be responsible for the differences in virulence (138). This presumption is supported by the observation that both CEACAM1a independent spread and neurovirulence map to a G310S substitution of the RBD of JHM.IA spike relative to JHM.SD spike, restoration of S310 with the JHM.IA spike confers a more neurovirulent phenotype and CEACAM1a independent spread (138). Expression of the JHM spike within the A59 background also confers the ability to spread in the absence of CEACAM1a (127), but replacement of the entire S1 subunit of A59 spike with that of JHM was not enough to confer CEACAM1a independent spread (154), suggesting that other regions of spike must influence the ability to carry out to CEACAM1a independent spread (154). Another example of the importance of the RBD for differences in pathogenesis is that a Q159L mutation within the RBD of A59 spike eliminates the ability to infect the liver, but not the brain (95-96).

Another region of spike that influences virulence is the hypervariable region (HVR). The longer HVRs found in the JHM strains JHM.SD (20), JHM.IA (138), JHM cl-2 (187) and JHM-DL (198) are associated with high neurovirulence. JHM.SD has a long HVR and is highly neurovirulent, while the A59 HVR has a 52 aa in frame deletion; however, replacement of the HVR of A59 with that of JHM.SD was not sufficient for enhance neurovirulence (154). The long HVR appears to be necessary for cell-to-cell fusion and spread in the absence of CEACAM1a (20, 51-52). The S1/S2 association of spikes with a long HVR is less stable, so that spike is more easily triggered to undergo fusion; the instability of spike is thought to be required for CEACAM1a independent spread (51, 81). Single amino acid deletions or mutations in HVR can lead to important changes in virulence, such as the attenuation observed with V5A13.1, an antibody escape mutant of JHM.SD (20, 44, 54, 154).
Heptad repeat (HR) domains also contribute to pathogenic potential important regions of the spike protein. Amino acid substitutions within HR1 of JHM (position 1114) are associated with different phenotypes depending on the substitution. An L1114R mutation is associated with neuroattenuation and restriction of replication to the olfactory bulb of the JHM.SD OBLV60 mutant (53, 146, 189). A L1114F substitution was found in both the glial tropic JHM-DL mutant JHM V-1 (199) and the attenuated soluble receptor-resistant JHM cl-2 mutant srr7 (166-167). The L1114F substitution was also associated with loss of neuron tropism (9, 198). Both L1114 substitutions are associated with the inability to induce CEACAM1a-independent cell fusion and with neuroattenuation (120-121, 185).

Spike contains the two known major H-2b CD8 T cell epitopes and also contains the major neutralizing B cell epitopes. The immunodominant CD8 T cell epitope is composed of amino acid 510-519; this epitope is unique to JHM spikes with long HVRs as it is within the deleted portion of the A59 HVR. The subdominant epitope is located at amino acid 598-603 within the JHM spike and is also encoded by A59. Although the major CD8 T cell epitope is deleted in A59, it induces a strong T cell response in the CNS, while JHM.SD, which has the major epitope, induces a weak T cell response in the CNS (72, 111, 164).

**Small Envelope Protein (E):** E is an integral membrane protein (220), and while it is nonessential in MHV, without it replication is highly reduced (84). E is targeted to the Golgi membrane where it is thought to be important to viral assembly and budding (194), and mutations in E can lead to misshapen virions, suggesting that it plays an important role in morphogenesis (46). The ability of E to form an ion channel has been speculated to be involved in assembly and morphogenesis (206). MHV and SARS-CoV E are pro-apoptotic (2). Evidence suggests SARS-CoV E may disrupt ion homeostasis in the cell, which could lead to apoptosis by induction of membrane depolarization (152).
Membrane Protein (M): M is an O-glycosylated, transmembrane protein. It has a short external N-terminal domain, three transmembrane domains, and an internal C-terminal tail. M is targeted to the Golgi membrane where it is believed to be essential to viral assembly. M is responsible for the inclusion of viral proteins and genomes into the virion through binding of S, HE, and RNA bound N.

Nucleocapsid Protein (N): N is a basic RNA binding protein (3, 175) that complexes with viral genome to form the helical nucleocapsid (180)). N of MHV has three conserved regions (I, II, and III) separated by two hypervariable regions (A and B) (142). Crystal structures of the related infectious bronchitis virus (IBV) and severe acute respiratory syndrome coronavirus (SARS-CoV) N show that N has two structured domains designated the N-terminal domain (NTD), beginning within conserved region I and ending in conserved region II, and the C-terminal domain (CTD), residing within conserved region II and ending before the hypervariable region B (43, 70, 73, 168, 219). Conserved region II is involved in RNA binding (118, 134), while conserved region III is involved in M binding (71).

Several functions have been attributed to N. N is important for viral assembly through its interaction with membrane protein (71), and N forms a protective coat for viral RNA. N associates with subgenomic mRNA as well as genomic RNA (5), and is important for virus replication (61) and transcription (19). While not required, N enhances recovery of infectious virus from transfected genome-length synthetic RNA (5, 218). It has also been suggested that N plays a role in translation of viral mRNA (188). Additionally, N of JHM associates with microtubules in a neuronal cell line (145), suggesting a possible role of N in trafficking and axonal transport in neurons. N of A59 has been reported to antagonize type I interferon by blocking RNaseL activity (212). Furthermore N from A59 and the highly hepatotropic strain MHV-3, but not JHM, were shown to be responsible for the induction of fgl2 (33, 124, 136). N induces fgl2 through the transcription factor HNF4α (135). It has also been reported that N partially localizes to the nucleus of infected cells (210).
Hemagglutinin-Esterase Protein (HE): HE is a nonessential glycoprotein that forms a second smaller spike on the viral envelope of some, but not all MHV strains (216) (173, 215). HE has both sialic acid binding and acetylemesterase activity, and has been proposed to act as a second attachment factor. The acetylemesterase activity acts as a receptor destroying enzyme and allows for reversible attachment to sialic acid residues. It has been suggested that HE may play a role in determining tropism, although this has not yet been demonstrated for any MHV strain (213-214) Yokomori, 1993 #556}, and may aid in viral spread (76). Tissue culture passaging of strains, such A59, was shown to select for mutations that abolish HE expression (103, 172).

The importance of HE as a factor in neurovirulence was explored in experiments were the HE pseudogene of A59 was replaced with the full-length HE gene of MHV-S to generate the following viruses: rMHV-A59S-HE⁺, a virus expressing MHV-S HE; rMHV-A59S-HE⁻, a virus expressing MHV-S HE that lacks acetylemesterase activity; and rMHV-A59S-HE⁻ an isogenic control that does not express the HE polypeptide (103). All three viruses displayed similar replication patterns in vitro and in the CNS. However, rMHV-A59S-HE⁻ outcompeted rMHV-A59S-HE⁺ and rMHV-A59S-HE in tissue culture, and upon passaging rMHV-A59S-HE⁺ and rMHV-A59S-HE⁻ developed mutations in HE that prevented its incorporation into membranes. These data suggest that there is selective pressure against HE during passage in tissue culture, and that selection is against virus incorporation of the protein, not enzymatic activity, consistent with the finding that increased virulence did not require active esterase activity (75). A second similar set of viruses were generated that expressed JHM S in addition to MHV-S HE in the A59 background (rMHV-JHMS-HE⁺, rMHV-JHMS-HE⁻, and rMHV-JHMS-HE⁻)(75). Both rMHV-JHMS-HE⁺ and rMHV-JHMS-HE⁻ were more virulent and spread further in the CNS than rMHV-JHMS-HE⁻. These experiments combined suggest that HE works in combination with compatible S to enhance neurovirulence, and that this is dependent on binding to neuramic acid containing moieties and not acetylemesterase activity.
**Internal (I) Protein:** The I protein is a 23kDa, membrane associated protein that is encoded within the +1 reading frame of the nucleocapsid gene (45). Nearly all MHV strains express the internal protein, with JHM being a notable exception. An A59 strain that was mutated to abrogate expression of the internal protein (Alb110) had no major defects in replication in vitro or in the liver and CNS; however, Alb110 produced smaller plaques and may replicate to slightly lower titers in the liver (45). For these reasons it is unclear what impact, if any, internal protein has on pathogenesis, though it appears to be of minimal importance.

**Nonstructural Proteins – Polyproteins 1a and 1ab**

ORF1a encodes polyprotein 1a (pp1a), while a ribosomal frameshift that extends translation of ORF1a into and throughout ORF1b produces polyprotein 1ab (pp1ab). These polyproteins, which are highly conserved among coronaviruses, are cleaved into 16 proteins, named nonstructural proteins (nsp)1-16, not to be confused with ns2, ns4, and ns5a, which are the nonstructural proteins, unique to betacoronavirus groups A viruses, encoded by ORF2a, ORF4, and ORF5a. Experiments with chimeric recombinant viruses in which regions spanning ORF1 through ORF2a were exchanged between A59 and JHM.SD suggest that the major pathogenic differences between these strains are not due to pp1a, pp1ab or ns2, but rather to proteins, encoded in the 3’ one third of the genome. However, these experiments did not completely rule out the possibility that these proteins may play minor roles in pathogenic differences (131). The functions of many of the small proteins for which the polyproteins are processed into are not well understood, but a few have been shown to be important virulence determinants, and these are described below.

**Nsp1:** Nsp1 (p28) is the most N-terminal cleavage product of pp1a and pp1ab. An nsp1 mutant A59 (MHV-nsp1delta99) had no obvious defects in replication in vitro, but was attenuated in both ability to replicate in the liver and cause hepatitis (129, 224). SARS-CoV Nsp1 promotes the degradation of host mRNA (129). Nsp1 of both MHV and SARS-CoV were reported to inhibit synthesis and/or signaling activities of IFN-β (201) though it is uncertain if this is a direct affect or is indirect through the generalized degradation of host mRNA (9).
**Nsp3**: Nsp3 is the third cleavage product of pp1a and pp1ab. It is a 180-200kDa protein that has an ADP-ribose 1"-phosphatase (ADRP, X, or macro) domain and two papain-like protease (PLP) domains (59, 159-160). All coronaviruses have a conserved ADRP domain, but its function is unknown. The main function of PLPs are to serve as proteases for the processing of the polyproteins. SARS nsp3 has been demonstrated to have deubiquinating activity and to be a type I IFN antagonist (7, 201, 221). The PLP of SARS-CoV inhibits both IRF3 and NFKappaB pathways (30, 49). PLP-2 of MHV is analogous to SARS-CoV PLP (i.e. it has deubiquitinating activity) but it is unclear if MHV PLP-2 is also a type I IFN antagonist (49, 221). Interestingly an A59 virus with a mutation in the ADRP domain (N1348A) predicted to prevent enzymatic activity caused almost no hepatitis, though it was able to replicate to titers only slightly reduced relative to wild type virus (42).

**Nsp14**: Nsp14 is a 3'–5' exoribonuclease of the DEDD superfamily (126, 174), and it has a "proofreading" activity that increases the fidelity of viral transcription (40). A Y6398H mutation had no effect on in vitro replication, but was attenuating after intracranial inoculation (176).

**Nonstructural Proteins – group specific proteins**

All coronaviruses have their own unique subset of small nonessential proteins that make up the group specific proteins. For MHV ORF2a, ORF4, and ORF5a encode the group specific proteins ns2, ns4, and ns5a.

**Ns2**: Ns2 is the protein product of ORF2a. It is a 30kDa protein with homology to the 2H phosphoesterase superfamily and is predicted to have 1",2"-cyclophosphodiesterase (CPD) activity (174). It has been suggested that the CPD domain and the ADRP domain (from nsp3) could be involved in nucleotide processing (59, 174) in which the CPD converts ADP-ribose-1",2"-cyclic phosphate into ADP-ribose 1" phosphate and ADRP removes the phosphate to convert ADP-ribose 1"phosphate, into ADP-ribose (159-160). ADRP also binds to mono- and poly-ADP-
ribose implying that it may play a role in ribosylation of host cell proteins, which may promote apoptosis and necrosis (41). A deletion of ORF2a of JHM had no affect on replication in vitro (170), and had no phenotype after intracranial inoculation (personal communication J. Leibowitz and S. Perlman). A deletion of both the ORF2a gene and HE psudeogene of A59 was attenuating in vivo after intracranial inoculation despite the ability to replicate to titers similar to that of wild type virus in vitro (28). Mutations in either of two catalytic histidines of ns2 in A59 cause a large decrease in viral titers in the liver, but have no affect on neurovirulence or in vitro replictation (165). These same mutations rendered virus more sensitive to type I interferon in bone marrow derived macrophages, suggesting ns2 acts as a type I interferon antagonist (unpublished).

**Ns4:** Ns4 is encoded by ORF4. JHM expresses a full length ns4, while A59 ORF4 is disrupted and instead has two shorter ORFs (ORF4a and ORF4b) that are likely nonfunctional. ORF4 is nonessential for replication in vitro (204, 217) and viruses ablated for ORF4 expression display wild type levels of neurovirulence (139).

**Ns5a:** Ns5a is encoded by ORF5a and is nonessential for replication in vitro (217). Ns5a plays an important role in mediating interferon resistance. It was demonstrated with recombinant viruses that ablation of expression of A59 ORF5a conferred increased sensitivity to interferon and that interferon resistance could be restored by reintroduction of ORF5a activity (78).

**SUMMARY AND FOCUS OF THESIS**

We aimed to gain further insight into the genes responsible for pathogenic differences among MHV strains and the mechanisms responsible for these differences. We were particularly interested in elucidating the mechanisms underlying the striking differences in virulence between JHM.SD and A59. JHM.SD is highly neurovirulent, while A59 is weakly neurovirulent (92, 155). However, A59 is able to induce moderate to severe hepatitis while JHM.SD cannot replicate in
the liver (92, 155). A59 and JHM.SD are approximately 94% homologous, which in a 31kb genome results in numerous sequence differences. A59/JHM recombinants in which the replicase genes were exchanged suggested that they were not responsible for major pathogenic differences (131). The spike protein had previously been demonstrated to be an important virulence factor; however differences in spike protein were unable to account fully for the differences between the virulence of A59 and JHM.SD (133, 164). This suggests that one or more of the following proteins must be important to virulence factors: HE, E, M, N, I, or ns5a. Here I explore the roles of N, I, and HE on pathogenesis.

To study the role of N in virulence we generated chimeric viruses in which the N gene was exchanged between A59 and JHM.SD. We also generated chimeric viruses where both the N and S genes were exchanged. S is a known virulence factor (72, 133, 155) and we wanted to determine how much of an affect S and N had together on virulence. We demonstrated that JHM N was able to enhance the virulence of A59, conferring an approximately 100 fold lower intracranial LD$_{50}$. The complementary chimeric virus, expressing the A59 N within the JHM.SD background, did not display an increased LD$_{50}$; however mice infected with this virus survived longer than JHM.SD. We demonstrated that JHM N was able to enhance replication and spread within the CNS compared to A59, but did not enhance spread in primary hippocampal neuronal cultures. A59 N expression, on the other hand, decreased replication and spread in the CNS relative to JHM.SD. Expression of both JHM S and N in the A59 background caused an even greater reduction in survival time and increased spread beyond that of S or N alone. The virus expressing A59 S and N in the JHM.SD background was attenuated and spread less in the CNS than expression of A59 S or N alone. JHM N also slightly reduced the induced T cell response when expressed in the A59 background while A59 N slightly increased the T cell response when expressed in the JHM.SD background. Additionally we demonstrated that neither expression of A59 N nor A59 N and S in the JHM.SD background conferred the ability to replicate in the liver. Expression of JHM N in the A59 background did not prevent the virus from replicating to high titer and causing severe hepatitis, but it did confer a slight reduction in titer.
We also examined the role of I protein. I protein is expressed by A59 but not JHM. Differences in pathogenesis between A59 and JHM.SD due to differences in I protein expression could complicate the results of the N experiments. Since I protein is encoded within the N gene, the N gene exchange viruses were also I gene exchanges. Previous reports suggested that I protein was not important to replication in vivo or in vitro, but virulence was not examined. We obtained an I⁻ mutant of A59 (Alb110) and a wild type control (Alb111) from Dr. Paul Masters. Alb111 and Alb110 were no different in virulence after intracranial or intrahepatic inoculation, suggesting that I protein is not very important to virulence, at least under these conditions. This also suggests that differences we observed in the N exchange viruses were properly attributed to N.

We also examined the role of HE in JHM.SD pathogenesis. This was of interest because JHM.SD expresses HE while A59 does not and previous experiments showed that MHVS HE could enhance virulence when expressed along with JHM S in the A59 background. We constructed a cDNA clone of JHM.SD that allowed us to easily manipulate the genome. This allowed us to generate an HE⁻ clone of JHM.SD. We were not able to detect any difference in neurovirulence either after intracranial or intranasal inoculation between the HE⁻ virus or a wild type control virus. While these observations do not prove that HE is unimportant to virulence, but they do suggest it is not a major virulence factor for CNS disease. The cDNA clone that was used for these experiments will be of great benefit to our lab and others in the field because it provides, for the first time for JHM.SD, a reverse genetics system that is both easy to use and allows manipulation of the entire genome.
REFERENCES


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Chapter Two

THE MURINE CORONAVIRUS NUCLEOCAPSID GENE IS A DETERMINANT OF VIRULENCE

ABSTRACT

The murine coronavirus, mouse hepatitis virus (MHV) strain A59, causes acute encephalitis and chronic demyelinating disease as well as hepatitis in mice. The JHM strain (also called MHV-4 or JHM.SD) causes fatal encephalitis and only minimal hepatitis. Previous analysis of chimeric recombinant MHVs in which the spike gene, encoding the protein that mediates viral entry and cell-to-cell fusion, was exchanged between JHM and A59 showed that the spike plays a major role in determining organ tropism and neurovirulence but that other genes also play important roles in pathogenic outcome. Here, we have investigated the role of the nucleocapsid protein in MHV-induced disease. The multifunctional nucleocapsid protein is complexed with the genomic RNA, interacts with the viral membrane protein during virion assembly, and plays an important role in enhancing the efficiency of transcription. A pair of chimeric recombinant viruses in which the nucleocapsid gene was exchanged between JHM and A59 was selected and compared to wild-type parental strains in terms of virulence. Importantly, expression of the JHM nucleocapsid in the context of the A59 genome conferred increased mortality and spread of viral antigen in the mouse central nervous system compared to the parental A59 strain, while having little effect on the induction of hepatitis. While the JHM nucleocapsid did not appear to enhance neuron-to-neuron spread in primary neuronal cultures, the increased neurovirulence it conferred may be due in part to the induction of a less robust T-cell response than that induced by strain A59.

INTRODUCTION

Coronaviridae are a family of large, single-stranded and positive-sense RNA viruses within the nidovirus superfamily. The murine coronavirus mouse hepatitis virus (MHV) is a
collection of strains with a wide range of tropisms, inducing neurological, hepatic, enteric, and respiratory diseases, with outcomes dependent upon the viral strain and the route of infection. Infection via intracranial (i.c.) or intranasal (i.n.) routes serves as a model for studying both acute and chronic virus-induced neurological diseases; these include models of encephalitis and the demyelinating disease multiple sclerosis. Two naturally occurring neurotropic strains, A59 and JHM, have been shown to induce very different pathologies following i.c. infection. The A59 strain is a weakly neurovirulent, tissue culture-adapted strain that induces mild encephalitis and moderate hepatitis (18, 38). A59 infection is cleared from the central nervous system (CNS) and liver following a robust CD8 T-cell response (20, 22, 47); however, viral RNA persists in the spinal cord, and chronic demyelination develops in animals surviving acute infection (11, 18, 24). In contrast, the JHM strain, which has been previously referred to as MHV-4 or JHM.SD (5, 34), is highly neurovirulent in weanling C57BL/6 (B6) mice, inducing fatal encephalitis in nearly all infected mice following inoculation with doses as low as 1 PFU. This enhanced virulence is attributed in part to its rapid spread in the CNS, which occurs by MHV receptor CEACAM1a-dependent and -independent mechanisms (26), and likely also to the lack of a robust CD8 T-cell response in the CNS (19).

We have previously selected chimeric A59/JHM recombinant viruses, which have been used to define the roles of both spike (S) and background genes in CNS pathogenesis. The S gene, encoding the protein responsible for attachment to the host cell and subsequent fusion and entry, as well as for cell to cell spread, is clearly a major determinant of MHV neurovirulence. Replacement of the S gene of A59 with that of JHM (S\text{JHM}) confers upon the recombinant A59 (rA59) virus a highly neurovirulent phenotype. This chimeric virus, rA59/S\text{JHM} is characterized by a 3-log_{10} decrease in the intracranial 50% lethal dose (LD_{50}), increased rate of viral antigen spread, and increased inflammation compared with wild-type rA59 (14, 21, 38). However, this chimeric virus is less neurovirulent than the wild-type recombinant JHM (rJHM) virus, likely due at least in
part to the induction of a robust CD8 T-cell response (14, 20). Furthermore, unlike rJHM, rA59/S_{JHM} induces hepatitis when inoculated at a high dose (31). Further analysis of additional A59/JHM chimeric viruses, including the reverse chimeric virus rJHM/S_{A59} (where the S gene of JHM has been replaced by that of A59) (31) and viruses with exchanges of 5' replicase gene portions of the genome (28), demonstrated that, in addition to S, one or more genes within the 3' end of the JHM genome are necessary for the extremely high neurovirulence of JHM.

The nucleocapsid protein (N), encoded in the most-3' gene of the MHV genome, plays several roles in infection. N is a basic RNA binding protein (1, 45) that plays structural roles by both complexing with genome RNA to form the viral capsid (46) and interacting with the viral membrane protein (M) during virion assembly (13). In addition, N has several other functions during replication. Nucleocapsid protein (i) associates with genomic and subgenomic mRNA (2); (ii) significantly enhances recovery of infectious virus from transfected genome-length synthetic RNA (2, 50); (iii) has been reported to associate with microtubules (37), suggesting a possible role for N in trafficking and axonal transport in neurons; and (iv) has been shown to antagonize type I interferon (IFN) by blocking RNase L activity (49). Furthermore, N proteins from A59 and a highly hepatotropic strain, MHV-3, but not JHM were shown to be responsible for the induction of fibrinogen-like protein 2 (fgl2), a multifunctional protein that has both procoagulant and immunosuppressive activities and leads to enhanced liver damage during MHV infection (6, 25, 33). Finally, N is unique among MHV structural proteins in that it is partially localized to the nucleus of infected cells (48).

N protein has three conserved regions (I, II, and III) separated by two hypervariable regions (A and B) (36). Crystal structures of the related infectious bronchitis virus (IBV) and severe acute respiratory syndrome coronavirus (SARS-CoV) N show that N has two structured domains designated the N-terminal
domain (NTD), beginning within conserved region I and ending within conserved region II, and the C-terminal domain (CTD), residing within conserved region II and ending before the hypervariable region B (7, 12, 16, 44, 51). Conserved region II is involved in RNA binding (23, 32) while conserved region III is involved in M binding (13). The N protein is highly conserved among MHV strains. Sequence analysis reveals 94% identity at the amino acid level between the N proteins of A59 and JHM and 28 amino acid differences, 11 of which are outside of the hypervariable regions. We have selected chimeric recombinant viruses in which the N gene alone or in combination with the S gene has been exchanged between A59 and JHM. These chimeric viruses have been used to investigate the role of N in virulence.

MATERIALS AND METHODS

Cells and viruses. Murine fibroblast (L2 and 17Cl-1) cells and feline Felis catus whole fetus (FCWF) cells were maintained in Dulbecco's minimal essential medium supplemented with 10% fetal bovine serum (FBS), 1% amphotericin B (Fungizone), 10 mM HEPES, and 2 mM L-glutamine. Primary neuronal cultures were generated from hippocampal tissue harvested from day 15 to 16 embryonic mice. Neurons were grown on poly-L-lysine-treated glass coverslips and cultured at 37°C with 5% CO₂ in neurobasal medium (Gibco) containing B27 supplement (Gibco). Neurons were allowed to differentiate for 4 days prior to infection (39).

Previously described viruses include the following: (i) wild types rA59 (38) and rJHM (31) and (ii) S exchange viruses rA59/SJHM (38) and rJHM/S459 (31). Chimeric viruses with
exchanges of N genes (rA59/N_{JHM} and rJHM/N_{A59}) or N and S genes (rA59/S_{JHM}/N_{JHM} and rJHM/S_{A59}/N_{A59}) were selected by targeted recombination as described below.

Recombinant viruses were propagated on mouse 17Cl-1 cells; plaque assays and plaque purification of recombinants were carried out on L2 cells (10). Wild-type rA59 and rJHM were indistinguishable in phenotype from their parental wild type (5, 21, 31, 38).

Please note that we have changed our previously used nomenclature in an effort to make it simpler and more logical. Recombinant viruses are labeled "r" for recombinant, followed by the strain from which the background genes are derived, followed by the strain of the genes that have been replaced. For example, rA59/S_{JHM} is a recombinant A59 expressing the JHM S; this virus was previously called SJHM/RA59. rJHM/S_{A59} was previously called SA59/RJHM.

**Plasmids.** For the selection of chimeric viruses of the A59 background, the plasmid pMH54 was utilized. pMH54 contains the 3' third of the A59 genome, from codon 48 of the hemagglutinin esterase (HE) gene through the 3' end of the genome (17). pSG6, a modification of pMH54, contains two silent point mutations at nucleotides 8838 and 8841, which generate a unique BspEI site after codon 444 of the N gene (10). This plasmid was kindly provided by Paul Masters.

The N gene of pSG6 was exchanged for that of JHM to create pSG6/N_{JHM}, the plasmid from which rA59/N_{JHM} was generated. Specifically, a fragment containing nucleotides 7529 to 9003 was amplified from pJHM (35) using PCR, *Pfu* polymerase, and primers that created 5' BssHII and 3' BspEI sites. The resulting fragment (beginning at codon 187 of the M gene and ending codon 445 of the N gene) spanning a conserved region of M and containing all the coding differences between the A59 and JHM N genes was cloned into pSG6, using unique 5' BssHII and 3' BspEI sites, to create pSG6/N_{JHM}. For selection of rA59/S_{JHM}/N_{JHM}, the A59 S gene was replaced by the JHM S gene using AvrII and SbfI sites as described previously (38).
The plasmid pJHM, analogous to pMH54, contains the 3' portion of the JHM genome (35) and was used to generate JHM background viruses. For selection of rJHM/N<sub>A59</sub>, pJHM was modified to create pJHM/N<sub>A59</sub>, by exchanging the N gene for that of A59 derived from pSG6. Thus, a silent mutation was introduced at nucleotide 7531 of pJHM to create a BssHII site at codon 187 of the M gene (corresponding to a site found in pMH54). PCR was then used to generate a fragment from nucleotide 7367 to 8898 of pSG6, which begins at codon 187 of the M gene and ends in the 3' untranslated region 30 nucleotides after the end of the N gene. Primers used to amplify this region contained a 5' BssHII site and created a 3' DraIII site, corresponding to a site present in pJHM. The BssHII and DraIII sites were used to clone the fragment into pJHM, creating pJHM/N<sub>A59</sub>. pJHM/N<sub>A59</sub> was further modified to create pJHM/S<sub>A59</sub>/N<sub>A59</sub> by exchanging the S genes using the AvrII and SbfI sites, as described above (38).

Selection of recombinant viruses. Targeted recombination was carried out as described previously ((17, 38). Briefly, FCWF cells were infected with fMHV-A59, a virus expressing the feline infectious peritonitis virus (FIPV) S within the A59 background, or with fMHV-JHM, a virus expressing FIPV S in the JHM background. RNAs transcribed in vitro from the chimeric plasmids described above were electroporated into infected FCWF cells, and infected transfected FCWF cells were overlaid onto confluent murine 17Cl-1 cells. Viruses were selected for their ability to infect murine cells and were plaque purified twice on L2 cells. At least two viruses of each genotype, isolated from independent recombination events, were plaque purified, and the S and N genes were sequenced. There were no differences observed between the in vitro and in vivo phenotypes of the two viruses of each genotype. Thus, we will show the data for one of each genotype virus.

Virus growth curves. Confluent monolayers of L2 cells were infected at a multiplicity of infection (MOI) of 1. At the times indicated in the figures, the cells and supernatants were
lysed by three freeze-thaw cycles, and debris was removed by centrifugation. Titers of lysates were then determined by plaque assay of L2 cells as previously described (10).

**Neuronal cultures, infections, and quantification.** Primary hippocampal neuronal cultures, as described above, were infected with the viruses indicated in the figure legends at a MOI of 1 or left uninfected. At days 1 to 4 postinfection, cultures were fixed in a solution of 2% paraformaldehyde in phosphate-buffered saline (PBS) containing 0.12 M sucrose and stored in PBS at 4°C until staining. Fixed neurons were stained with antinucleocapsid (anti-N) monoclonal antibody (MAb) 1.16.1 (kindly provided by J. Leibowitz, Texas A&M University) using a Vector ABC immunoperoxidase kit (Vector, Burlingame, CA). Cells were counterstained with either hematoxylin or methyl green. Quantification of the area of individual foci was carried out using the color cube-based segmentation function of Image Pro, version 5.0, software.

**In vivo infections. (i) Mice and inoculations.** Pathogen-free 4- to 5-week-old male C57BL/6 mice were obtained from NCI (Fredrick, MD). All experiments were performed in containment within a biosafety level 2 animal facility and conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) at the University of Pennsylvania.

(ii) Virulence assays. Virus was serially diluted 10-fold with PBS containing 0.75% bovine serum albumin. Mice (5 to 10 per viral dose) were anesthetized with isoflurane and inoculated in the left cerebral hemisphere with virus. Mice were monitored daily for 21 days and euthanized when they became moribund and counted along with mice found dead the following day. Statistical comparison of survival curves was performed using a Gehan-Breslow-Wilcoxon test, and LD_{50} values were calculated (40).

**Virus replication in vivo. (i) Brain.** Mice were infected i.c. with 50 PFU of virus and on day 5 postinfection were sacrificed and perfused with 10 ml of PBS. The brains were
removed and cut in half along the midline. The right halves were fixed in formalin to be used for histology as described below and the left halves were placed in 2 ml of isotonic saline containing 0.167% gelatin, weighed, and stored at −80°C for virus titration. Brains were homogenized, and virus titers were determined by plaque assay as described previously (10). Statistical comparisons were made using a two-tailed *t* test.

(ii) Liver. Mice were infected intrahepatically (i.h.) with 500 or 1.6 x 10⁴ to 1.8 x 10⁴ PFU of virus as described previously (30). Five days postinfection mice were sacrificed and perfused with PBS. Livers were harvested and homogenized, and titers of lysates were determined for infectious virus as described above. Statistical comparisons were made using a two-part two-tailed *t* test.

**Histology, immunohistochemistry, and quantification of viral antigen.** Formalin-fixed brains, harvested from mice sacrificed at 5 days postinfection, were paraffin embedded and sectioned sagitally. Viral antigen was detected using anti-N MAb 1.16.1 and the Vector ABC immunoperoxidase kit, as previously described (30). Sections were counterstained with hematoxylin. Pictures taken of each stained brain section were analyzed using the color cube-based segmentation function of Image Pro, version 5.0, software to calculate total stained area and total area of each section. The ratio of stained area to total section area was used as a means of quantifying the amount of viral antigen staining in each section. Five or more mice were used per group, with two adjacent sections per mouse. A two-tailed *t* test was used to determine significance.

Histology, immunohistochemistry, and quantification of antigen for the liver sections were carried out using the same methodology as above, utilizing a section of the median liver lobe cut about 0.5 cm from the tip. Hepatitis was scored by examining liver sections stained with hematoxylin and eosin. Sections were evaluated for degree of inflammation and necrosis and scored on a four point scale (4, severe; 3, moderate; 2, mild; 1, minimal; 0, none) as previously described (3, 31).
**Isolation of brain mononuclear cells.** Mice were infected either i.c. with 10 PFU of virus or i.n. with 500 PFU of virus. Seven days postinfection, the peak of T-cell response in the brain, mice were perfused, and brains were harvested (20). Brains were placed in RPMI medium containing 10% FBS and homogenized through a nylon mesh bag (pore diameter, 64 µm) with moderate pressure from a syringe plunger. Cells were then passed through a 30% Percoll gradient and through a cell strainer (pore diameter, 70 µm) and then washed and counted.

**T-cell quantification.** Up to 1 x 10⁶ brain-derived mononuclear cells were analyzed per brain. Cells were stained for surface expression of CD3, CD8, and CD4 using fluorescein isothiocyanate (FITC)-conjugated CD8α, clone 53-6.7; phycoerythrin (PE)-conjugated CD4, clone GK1.5) and stained for T-cell receptors specific for the major CD8 epitope, S510, using allophycocyanin (APC)-conjugated major histocompatibility complex (MHC) tetramers (kindly provided by Stanley Perlman, University of Iowa). Cells were fixed in 2% paraformaldehyde and analyzed using a FACSCalibur flow cytometer (Becton Dickinson). Total cell numbers per mouse were determined by multiplying the fraction of live cells positive for a given marker by the total number of live cells isolated. CD8 T cells were defined as those cells of lymphocyte size based on forward and side scatter that expressed CD3 and CD8 but lacked CD4 expression. S510-specific T cells were those within the CD8 T-cell population that were stained by the S510 tetramer. CD4 T cells were defined as those cells of lymphocyte size that expressed CD3 and CD4 but lacked CD8 expression.

For quantification by intracellular IFN-γ staining assay, similar methodology was used as above except that peptide incubation and intracellular IFN-γ staining steps were added in place of the MHC tetramer staining (27, 39), and CD3 staining was omitted. Thus, cells
were cultured with 10 U of human recombinant interleukin-2 and 1 µl/ml brefeldin A (Golgiplug; BD Biosciences) in the presence of 1 µg/ml of peptide corresponding to the subdominant CD8 T-cell epitope, S598, in RPMI 1640 medium supplemented with 5% FBS for 5 h at 37°C. Cells were then stained for the surface expression of CD4 and CD8. These cells were then fixed and permeabilized using a Cytofix/Cytoperm kit (BD Biosciences) and stained for IFN-γ with an FITC-conjugated monoclonal antibody (BD Pharmingen).

RESULTS

Selection and in vitro characterization of A59/JHM chimeric recombinant viruses with exchanges of nucleocapsid genes. Previous analyses of chimeric A59/JHM recombinant viruses have shown that the differences in pathogenesis between the highly neurovirulent JHM and the weakly neurovirulent but hepatotropic A59 strains are in part attributed to the S gene. However, it is clear that the extremely high neurovirulence of JHM, the inability of JHM to induce a robust CD8 T-cell response, and the inability of JHM to induce hepatitis are influenced by other genes encoded within the 3′ third of the genome, which includes the structural genes envelope (E), membrane (M), nucleocapsid (N), and internal (I) and the putative nonstructural genes ORF4 and ORF5a (14, 29, 31). Because of the multiple functions of N both as a structural protein and in replication, we have investigated its contributions to pathogenesis. Targeted recombination was used to select isogenic viruses differing only in the N gene. More specifically, we selected A59 background viruses in which the A59 N gene was replaced with that of JHM (rA59/N<sub>JHM</sub>). We also selected the reverse JHM background viruses, replacing the N gene of JHM with that of A59 (rJHM/N<sub>A59</sub>) (Fig. 2-1). To investigate to what extent phenotypic differences between JHM and A59 can be explained by S and N together, a second set of chimeric
viruses was selected in which both the S and N genes were exchanged (Fig. 2-1). Also shown in Fig. 2-1 are representations of the genomes of previously described recombinants in which S genes have been exchanged (31).

Figure 2-1. Schematic representation of the virions and corresponding genomes of recombinant viruses. Shown on the left are A59 background viruses and, on the right, the JHM background viruses. A59 components are in white, and JHM components are in black. The positions of the spike (S) and nucleocapsid (N) genes are noted. The 5’ replicase gene is not shown to scale, as indicated by hash marks.

To verify that the chimeric viruses replicate efficiently in tissue culture and to determine if the genotype of N has an effect on replication patterns, rA59/N\textsubscript{JHM} and rJHM/N\textsubscript{A59} were compared to parental wild-type rA59 and rJHM in one-step growth curves (Fig. 2-2A). As previously reported, rJHM replicates with significantly slower kinetics and to a lower titer than rA59 in tissue culture (38). The N exchange viruses replicated with similar kinetics as their respective parental viruses although rJHM/N\textsubscript{A59} replicated slightly less efficiently than rJHM (Fig. 2-2A). Similarly, one-step growth curves were performed using rA59/S\textsubscript{JHM}/N\textsubscript{JHM} and rJHM/S\textsubscript{A59}/N\textsubscript{A59} and their parental controls rA59/S\textsubscript{JHM} and rJHM/S\textsubscript{A59} (Fig. 2-2B). As previously reported, the \textit{in vitro} replication patterns of chimeric viruses segregated with the S gene (38). Importantly, exchange of the N genes did not alter either the kinetics of replication or the final extent of replication \textit{in vitro} (Fig. 2-2B). These data indicate that these chimeric recombinants are not impaired in the ability to replicate \textit{in}
vitro. In addition, plaques of chimeric viruses were similar in size and shape to the corresponding isogenic parental viruses (data not shown).

**Figure. 2-2: Replication of recombinant viruses.** L2 cells were infected (in duplicate) at an MOI of 1 with wild-type (A) and N exchange viruses or with S exchange viruses with and without N exchange (B). At the times indicated, virus was harvested from combined supernatants and cells, and titers were determined on L2 cell monolayers as described in Materials and Methods. Duplicate samples were averaged. The data are from one representative experiment of two.

**JHM nucleocapsid protein is a determinant of high neurovirulence.** rA59 and rJHM, like the wild-type isolates from which they were derived, display strikingly different levels of virulence in the CNS (14, 21). Intracranial (i.c.) infection with less than 10 PFU of rJHM typically kills all infected mice. In contrast, i.c. infection with rA59 requires approximately 3 x 10^3 to 5 x 10^3 PFU to kill half the mice (14, 21). JHM S greatly contributes to this difference as rA59/S_{JHM} also kills mice with fewer than 10 PFU (14, 21, 38). The rJHM-infected mice, however, die more quickly than the rA59/S_{JHM}-infected mice, with the mean
survival time being about 2 days less for JHM-infected mice (14). To determine if JHM N contributes to high neurovirulence, weanling C57BL/6 mice were infected i.c. (at the doses indicated in Fig. 2-3) with rA59/N<sub>JHM</sub> and rJHM/N<sub>A59</sub> and their parental viruses rA59 and rJHM. Mice were observed daily for illness and mortality; survival times were recorded, and LD<sub>50</sub> values were calculated. Consistent with previous observations, rA59 was not lethal at a dose of 500 PFU (Fig. 2-3A) and the LD<sub>50</sub> averaged 7.7 x 10<sup>3</sup> (Table 2-1). In contrast, infection of mice with only 10 PFU of rA59/N<sub>JHM</sub> killed 60 to 80% (Fig. 2-3A and C) of mice, and the survival curve was similar to that of infection with 5 x 10<sup>3</sup> PFU of rA59 (Fig. 2-3A). The LD<sub>50</sub> for rA59/N<sub>JHM</sub> was <10 PFU, 100- to 1,000-fold less than that of rA59 and similar to that of rJHM (Table 2-1). Thus, expression of JHM N within the rA59 background enhances neurovirulence significantly. Conversely, expression of A59 N from within the rJHM background (rJHM/N<sub>A59</sub>) decreased neurovirulence to a slight extent compared with rJHM (Fig. 2-3B). This difference is difficult to measure, probably because of the very high virulence (LD<sub>50</sub> of <10 PFU) of both of these viruses.

To determine to what extent the neurovirulence differences between A59 and JHM can be explained by a combination of the N and S genes, mice were infected with chimeric viruses (rA59/S<sub>JHM</sub>, rA59/N<sub>JHM</sub>, rA59/S<sub>JHM</sub>/N<sub>JHM</sub>, rJHM/S<sub>A59</sub>, and rJHM/S<sub>A59</sub>/N<sub>A59</sub>) and the parental rA59 and rJHM at the doses specified in Fig. 2-3. As previously observed, rA59/S<sub>JHM</sub> is highly lethal at 10 PFU, which indicates that it is significantly more virulent than rA59 (Fig. 2-3C) (14, 38). Infection with a virus expressing both the JHM S and N genes within the rA59 background (rA59/S<sub>JHM</sub>/N<sub>JHM</sub>) was highly lethal, resulting in survival kinetics close to that of rJHM (Fig. 2-3C). Expression of the A59 S in the JHM background increased the LD<sub>50</sub> to a value similar to that of rA59, which in this experiment was 9.5 x 10<sup>3</sup> PFU for rJHM/S<sub>A59</sub> and 1.2 x 10<sup>4</sup> PFU for rA59 (Fig. 2-3D). Interestingly rJHM/S<sub>A59</sub>/N<sub>A59</sub> was nonlethal even at the very high dose of 3 x 10<sup>5</sup> PFU, indicating that replacing the JHM N gene with that of A59 is attenuating (Fig. 2-3D and Table 2-1). rA59 when expressing JHM S and N closely resembles rJHM in terms of virulence, but
expressing both A59 S and N in the rJHM background attenuates the virus beyond the level of A59.

**Figure 2-3: Survival curves of infected 4-week-old C57BL/6 mice.** Mice were infected with recombinant viruses at the doses indicated and observed over 21 days for mortality. (A) Infection with rA59 (500 PFU and 5 x 10³ PFU) and rA59/N_{JHM} (10 PFU, 100 PFU, and 1 x 10³ PFU). rA59/N_{JHM} at a dose of 100 PFU is significantly more virulent than rA59 at a dose of 500 (n = 5 each; P = 0.0155). (B) Infection with rJHM and rJHM/N_{A59} at 10 PFU. rJHM/N_{A59} is significantly less virulent than rJHM (n = 10 and 5, respectively; P = 0.0272). (C) Infection with rJHM, rA59/N_{JHM}, rA59/S_{JHM}, and rA59/S_{JHM}/N_{JHM} (10 PFU). rA59/S_{JHM}/N_{JHM} is more virulent than rA59/N_{JHM} (n = 10 each; P = 0.0030) and rA59/S_{JHM} (n = 10 and 9, respectively; P = 0.0016), and rJHM is more virulent than rA59/S_{JHM}/N_{JHM} (n = 10 each; P = 0.0319). (D) Infection with rJHM/S_{A59}/N_{A59} (3 x 10⁵ PFU) and rJHM/S_{A59} (3 x 10³ PFU, 3 x 10⁴ PFU, and 3 x 10⁵ PFU). rJHM/S_{A59} is more virulent than rJHM/S_{A59}/N_{A59} (n = 5 each; P = 0.0041). The data are from one representative experiment of two or more.

<table>
<thead>
<tr>
<th>Virus</th>
<th>LD₅₀ (PFU)</th>
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<tbody>
<tr>
<td>rA59</td>
<td>7.7 x 10³</td>
</tr>
<tr>
<td>rA59/N_{JHM}</td>
<td>&lt;10</td>
</tr>
<tr>
<td>rA59/S_{JHM}</td>
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<td>rA59/S_{JHM}/N_{JHM}</td>
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<td>rJHM</td>
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<tr>
<td>rJHM/N_{A59}</td>
<td>&lt;10</td>
</tr>
<tr>
<td>rJHM/S_{A59}</td>
<td>9.5 x 10³</td>
</tr>
<tr>
<td>rJHM/S_{A59}/N_{A59}</td>
<td>&gt;3.0 x 10⁵</td>
</tr>
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Table 2-1. LD50 values

Replication and spread of recombinant viruses in the CNS. Despite vastly different levels of neurovirulence, wild-type and A59/JHM spike exchange chimeric recombinants replicate to similar levels in the brain during the first week postinfection, with titers and viral antigen levels peaking at day 5 (31, 38). Differences in neurovirulence are reflected in the extent of viral antigen detected in the brain, with rJHM-infected mice displaying extensive antigen spread compared to rA59-infected mice and with chimeric rA59/SJHM-infected mice displaying an intermediate level, consistent with neurovirulence as described above (14, 20, 39) (Fig. 2-3C). Thus, we compared both virus titer and extent of viral antigen expression in the brains of mice infected with wild-type chimeric recombinant viruses at day 5 postinfection. Mice were infected with 50 PFU of chimeric viruses rA59/NJHM and rJHM/NA59 and parental viruses rA59 and rJHM. At 5 days postinfection, brains were harvested and titrated for infectious virus by plaque assay. All viruses replicated to high titers in the brain (Fig. 2-4). However, exchange of the N genes appeared to affect replication in the brain, as demonstrated by a slight, but reproducibly significant, increase in rA59/NJHM replication over rA59 (P = 0.0001) (Fig. 2-4) and a similarly small decrease in replication of rJHM/NA59 compared to rJHM (P = 0.0128) (Fig. 2-4).

To determine the extent of virus spread in the brain at the peak of acute infection, a consistent correlate of neurovirulence, mice were infected with 50 PFU of rA59/NJHM and rJHM/NA59 as well as the parental viruses rA59 and rJHM. Sagittal sections from brains harvested at 5 days postinfection were stained for viral antigen.Brains from rA59/NJHM-infected mice expressed more antigen than those from rA59-infected mice but less than rJHM-infected mice (Fig. 2-5A). Similar to infection with rA59/NJHM and as expected (14, 38), rA59/SJHM-infected mice displayed levels of antigen intermediate between infections with parental rA59 and rJHM (Fig. 2-5A). When JHM S and N genes are both expressed
in rA59/S_{JHM/N_{JHM}}, the virus expresses antigen to an even greater extent, closer to that observed for rJHM (Fig. 2-5A). These observations were confirmed by quantification of antigen staining, carried out using Image Pro software as described in Materials and Methods (Fig. 2-5C). A similar pattern was observed in spread of the JHM background viruses. The extent of antigen was reduced in brain sections from rJHM/N_{A59}-infected mice compared to those from rJHM-infected mice, and the extent of antigen observed in sections from rJHM/S_{A59}-infected mice was reduced further (Fig. 2-5B and D). Finally, sections from mice infected with rJHM/S_{A59}/N_{A59} had even less antigen expression, similar to rA59-infected mice (Fig. 2-5B and D).

**Figure 2-4: Replication of chimeric viruses in the brain.** C57BL/6 mice (four or more per virus) were inoculated i.c. with 50 PFU of the indicated viruses. At 5 days postinfection mice were sacrificed, and virus titers from brain lysates were determined by plaque assay on L2 cells. Symbols represent individual animals, and the lines represent the mean and the standard error. *, \( P = 0.01 \); **, \( P = 0.0001 \). Data are from one representative experiment of two or more.

**Exchange of nucleocapsid protein in A59/JHM chimeric viruses does not affect spread of MHV in primary neuronal cultures.** To begin to understand the mechanism by which JHM N enhances neurovirulence and spread in the CNS in vivo, we tested the hypothesis that JHM N may enhance the ability to spread among neurons, the major CNS cell type infected by both JHM and A59 (8, 26) (unpublished data). JHM N was found to be closely associated with microtubules in the rat neuronal cell line OBL-21 and to share
homology with microtubule binding protein, tau; this implies a possible role of N in microtubule transport and spread (37). Thus, we compared the spread of wild-type viruses and N exchange chimeras in primary neuronal cultures by examining the sizes of discrete infected foci (Fig. 2-6A). We had previously observed that rA59 and rJHM initially produced similar numbers of infected foci in primary neurons but that, by 24 to 48 h postinfection, the rJHM-infected cultures have larger numbers of cells per focus (unpublished data). Neurons were infected at an MOI of 1 with rA59/N_JHM and rJHM/N_A59 along with control viruses rA59 and rJHM, and cultures were fixed and stained with MAb for N expression at 1 and 3 days postinfection (Fig. 2-6A) or 2 and 4 days postinfection (Fig. 2-6B). (The rA59- and rA59/N_JHM-infected cultures were assayed at later times because the infection spreads more slowly than in rJHM-infected cultures). No obvious differences in spread were observed; isolated foci of rA59/N_JHM-infected cells were similar in size to those generated by infection with rA59, and foci of rJHM/N_A59-infected cells were similar in size to those generated by rJHM infection (Fig. 2-6A and B). Quantification of the area of antigen staining per focus using Image Pro software confirmed that there were no significant differences in in vitro spread between isogenic viruses differing only in N (Fig. 2-6C and D). It has previously been observed that expression of JHM S within the A59 background conferred enhanced spread in primary neuronal cultures as measured by the numbers of cells per infected focus (39). When JHM S and N were expressed together in the A59 background, N did not enhance spread (data not shown). Thus, while expression of JHM N within the A59 background does seem to enhance CNS spread in vivo, this does not appear to be due to an inherent ability to spread more rapidly among neurons.
Figure 2-5: Viral antigen expression in the brains of infected mice. C57BL/6 mice were inoculated i.c. with 50 PFU of virus and sacrificed at 5 days postinfection. Brains were harvested, sectioned sagitally, and stained for viral antigen expression with anti-N MAb as described in Materials Methods. (A) Frame i, rA59; frame ii, rJHM; frame iii, rA59/NJHM; frame iv, rA59/SJHM; frame v, rA59/SJHM/NJHM. (B) Frame i, rA59; frame ii, JHM; frame iii, rJHM/NA59; frame iv, rJHM/SA59; frame v, rJHM/SA59/NNA59. Panels C (A59 background viruses from panel A) and D (JHM background viruses from panel B) show quantification of antigen staining using the color-cubed segmentation function of Image Pro, version 5.0, software. The y axis represents the area of antigen stain relative to rJHM, which is set to 100. Data shown represent the mean and standard error and are from one representative of two or more experiments. *, $P < 0.05$; **, $P \leq 0.0001$; #, $P = 0.08$. 

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Induction of a robust CNS T-cell response by MHV is modified by the exchange of A59 and JHM nucleocapsid genes. rA59 infection induces a strong CD8 T-cell response in the CNS, and this response is crucial for clearance of virus (11, 19). In contrast, the CD8 T-cell response against rJHM in the CNS is minimal (14, 19, 41). The lack of an effective CD8 T-cell response likely contributes to the high neurovirulence of JHM. Interestingly, the extent of the CD8 T-cell response is not dependent on the S gene but, rather, on one or more background gene(s), as evidenced by the finding that rA59/SJHM induces an even stronger T-cell response than rA59 while rJHM/S_{A59}, like rJHM, fails to induce a significant T-cell response (14, 19). We used the N exchange chimeric viruses to investigate whether N protein is a determinant of the extent of T cell response in the CNS.

For studies of CD8 T-cell response, we compared N exchange viruses to isogenic parental viruses. Initially, we used as parental controls viruses expressing the JHM S because the immunodominant MHV CD8 T-cell epitope, S510 (H-2b), is not expressed by the A59 S protein, and we wanted to compare viruses with the same CD8 T-cell epitopes. Mice were infected i.n. with 500 PFU of rJHM/N_{A59} or rJHM and, as a positive control for CD8 T-cell response, rA59/SJHM. At 7 days postinfection brain mononuclear cells were isolated and stained for expression of CD3-, CD4-, CD8-, and S510-specific T-cell receptors. Flow cytometry was used to determine the percentage of CD4, CD8, and S510 epitope-specific CD8 T cells within each sample, and the T-cell numbers per brain were calculated by multiplying the percentages of CD8, CD4, and specific CD8 T cells by the total number of cells isolated from each brain (Fig. 2-7A). The T-cell response (both CD8 and CD4) to rJHM/N_{A59} was significantly greater than the response to rJHM but less than that to rA59/S_{JHM}. Thus, JHM N was required in order for the T-cell response against the virus to be weak. Similar analyses of the reciprocal JHM background viruses were carried out to investigate whether expression of the JHM nucleocapsid in the A59 background confers a less robust T-cell response phenotype. Mice were infected with rA59/S_{JHM}/N_{JHM}. 

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rA59/S<sub>JHM</sub>, and rJHM, and the total numbers of CD8<sup>-</sup>, CD4<sup>-</sup>, and S510-specific CD8 T cells in the brains of infected mice were calculated as above (Fig. 2-7B). Brain mononuclear cells from rA59/S<sub>JHM</sub>-infected mice contained more CD8<sup>-</sup>, CD4<sup>-</sup>, and S510-specific CD8 T cells than those from rA59/S<sub>JHM</sub>/N<sub>JHM</sub>-infected mice. Cells from the brains of rJHM-infected mice had, as expected, minimal levels of CD8<sup>-</sup>, CD4<sup>-</sup>, and S510-specific CD8 T cells, significantly less than brains of rA59/S<sub>JHM</sub>-infected mice. Thus, expression of the JHM N did reduce the T-cell response compared with the isogenic parental virus expressing A59 N; however, the difference did not reach statistical significance.

To determine the level of T-cell expression conferred by expression of the JHM N protein alone in the presence of A59 background genes, mice were infected with rA59 and rA59/N<sub>JHM</sub>, and the total numbers of T cells were quantified as above. Since we were unable to obtain a suitable MHC tetramer reagent for quantification of S598-specific T cells, we quantified S598-specific T cells in the brains of infected animals utilizing an intracellular cytokine staining assay for IFN-γ, following incubation with S598 peptide (Fig. 2-7C). Brain mononuclear cells from rA59-infected mice contained more total CD8- and S598-specific CD8 T cells than generated by rA59/N<sub>JHM</sub> infection. As shown in Fig. 2-7A, the difference in the numbers of total CD8 T cells was statistically significant. Thus, the data in Fig. 2-7 indicate that expression of the A59 N in the JHM background leads to an increase in the T-cell response while expression of JHM N from within the rA59 genome likely leads to a small reduction in the T-cell response.
Expression of JHM nucleocapsid protein from within the A59 background does not impair the ability of MHV to induce hepatitis. In addition to the striking difference in neurovirulence, rJHM and rA59 differ greatly in ability to infect the liver and cause hepatitis. While rA59 induces moderate hepatitis when inoculated directly into the liver at a dose of 500 PFU, JHM replicates only minimally in the liver even when doses as high as $10^5$ PFU are administered. Perhaps surprisingly, analysis of chimeric viruses indicates that the ability to induce hepatitis does not map to the A59 S gene but, rather, to another gene(s) encoded in the 3' end of the genome (31). In addition, the N genes of some MHV
strains were shown to be responsible for the induction of fgl2, a procoagulant, which was necessary for MHV-3-induced fulminant hepatitis in BALB/c mice (6, 25, 33). Thus, we used the A59/JHM N exchange viruses to further investigate a role for N in replication and virulence in the liver. Mice were infected intrahepatically, a route which induces hepatitis but no CNS infection, with 500 PFU of rA59, rJHM, and rA59/N_JHM. Both rA59 and rA59/N_JHM replicated to high titers that were significantly greater than the titer of rJHM, which replicated near or below the limit of detection (Fig. 2-8A). Thus, JHM N expression does not preclude replication in the liver though rA59/N_JHM replicated to a slightly but significantly lower titer than rA59 (P value of 0.0015). Replication in the liver of JHM background chimeras rJHM/N_A59 and rJHM/S_A59/N_A59 was also compared to that of parental rJHM and rA59 (Fig. 2-8B). Because rJHM replicates to such a minimal extent in the liver, very large inocula (1.8 x 10^4 PFU) were used. All of the JHM background viruses replicated near or below the limit of detection, despite the difference in amount inoculated. There were no significant differences between rJHM/N_A59, rJHM/S_A59/N_A59, and rJHM in the levels of replication, so neither A59 N nor A59 S and N in combination is able to confer upon rJHM the ability to replicate in the liver.
Figure 2-7. T-cell response to recombinant viruses in the brain. Mice were infected with recombinant viruses and sacrificed at 7 days postinfection. Mononuclear cells were isolated from the brains of infected animals, stained with T-cell type-specific antibodies and either S510-specific tetramers (A and B) or assayed for secretion of IFN-γ in response to S598 peptide (C), and analyzed by flow cytometry as described in Materials and Methods. The total number of each T-cell population in the brain was determined by multiplying the percentage of each cell type by the total number of cells isolated per brain. Shown are the means and standard errors for the total CD8 T, epitope-specific CD8 T, and CD4 T cells in the brains of mice infected with rJHM/N_{ASS}, rA59/S_{JHM}, and rJHM (A); rA59/S_{JHM}/N_{JHM}, rA59/S_{JHM}, and rJHM (B); and rA59 and rA59/N_{JHM} (C). *, P < 0.05; **, P < 0.0005; #, P = 0.15; ##, P ≤ 0.09. Each panel is representative of at least two experiments.

Liver sections from a similar experiment were analyzed for degree of hepatitis and viral antigen expression. To quantify hepatitis, sections were stained with hematoxylin and eosin, observed for necrosis and immune infiltration, and blindly scored for hepatitis.
severity, using a four-unit scoring scale (4, severe; 3, moderate; 2, mild; 1, minimal) (3, 31). Liver sections from rA59- and rA59/N\textsubscript{JHM}-infected mice displayed moderate to severe hepatitis, with average scores of 3.75 and 3.31, respectively (Fig. 2-9A). The levels of hepatitis were similar for infections with rA59 and rA59/N\textsubscript{JHM}, indicating that in the context of A59/JHM chimeras, N is not an important determinant of hepatovirulence. Sections from mice infected with a very high dose of rJHM (approximately 35-fold more than the other viruses) displayed little visible damage; however, they did exhibit some inflammation (Fig. 2-9A). To measure viral antigen, sections were immunoperoxidase stained using anti-N MAb as above (Fig. 2-9B). Sections from both rA59- and rA59/N\textsubscript{JHM}-infected mice had extensive staining, with rA59 sections showing slightly more antigen. Minimal staining was observed in liver sections from mice that had been inoculated with a high dose of rJHM. Image Pro software was utilized to measure the extent of viral antigen staining. Antigen levels in the liver from rA59/N\textsubscript{JHM}-infected mice were not significantly different from those of rA59-infected mice while livers from rJHM-infected mice, as expected, had minimal levels of viral antigen (Fig. 2-9C).

DISCUSSION

The availability of a reverse genetics system for MHV and the selection and characterization of A59/JHM chimeric recombinant viruses have allowed us to begin to map the viral genes responsible for the strain-specific differences in pathogenesis.

Previous studies of chimeric A59/JHM recombinant viruses indicated that (i) the JHM S is a determinant of neurovirulence, but not the only determinant (Phillips, 1999 #1782); (ii) other viral genes encoded in the 3’ portion of the genome contribute significantly to the extensive antigen spread in the CNS and high mortality induced by JHM (14, 21, 31, 38); and (iii) the ability to induce hepatitis maps not to the A59 spike but, rather, to other
genes encoded in the 3' portion of the viral genome (28, 31). Because of the multifunctional nature of N protein, with roles as a structural protein and in replication, we chose to examine the role of N in pathogenesis. Thus, we selected and characterized A59/JHM chimeric viruses in which the N genes were exchanged. The ability to obtain clear answers in mapping pathogenic properties both in our previous studies (28, 31, 38) and in the studies described herein indicates that the use of chimeric viruses is not generally compromised by phenotypes that may be due to interactions of heterologous proteins.

Figure 2-8: Replication of chimeric viruses in the liver. C57BL/6 mice were inoculated intrahepatically with viruses at the doses as indicated. Mice were sacrificed at 5 days postinfection, and virus titers were determined from liver lysates. (A) rA59, rJHM, and rA59/N_{JHM} (500 PFU). (B) rA59 (500 PFU) and rJHM, rJHM/N_{A59}, and rJHM/S_{A59}/N_{A59} (1.6 x 10^4 PFU). Symbols represent individual animals, and the lines represent the mean and the standard error. The dotted lines represent the limits of detection. These data are from one representative experiment of two or more. *, P < 0.02; **, P < 0.0001.
Figure 2-9: Pathology and viral antigen expression in the livers of mice infected with chimeric viruses. C57BL/6 mice were inoculated intrahepatically with 500 PFU of rA59 and rA59/N_{JHM} and 1.8 x 10^4 PFU of rJHM. Mice were sacrificed at 5 days postinfection. Livers were harvested, sectioned, and stained either for pathology or viral antigen expression. (A) Hematoxylin- and eosin-stained sections: frame I, rA59; frame ii, rA59/N_{JHM}; frame iii, rJHM. (B) Immunoperoxidase-stained sections using anti-N MAb: frame i, rA59; frame ii, rA59/N_{JHM}; frame iii, rJHM. (C) Quantification of antigen staining using the color cube-based segmentation function of Image Pro, version 5.0, software. The y axis represents the area of antigen stain over total area of the liver section, and values are shown as mean and standard error. The data shown in all panels are representative of four or five mice per group; two adjacent sections were stained per animal. *, P < 0.05.
When N exchange recombinant viruses were compared to their respective parental wild-type viruses, there were no major differences observed in plaque morphology (data not shown) or in replication in L2 cells (Fig. 2-2A). In contrast, in vivo differences were striking. JHM N, when expressed within the A59 background (rA59/N
\text{JHM})\text{A59}, conferred enhanced virulence, as evidenced by an LD$_{50}$ approximately 1,000-fold lower than that of rA59. The reverse exchange viruses showed a similar but less dramatic result; expression of A59 N within the JHM background (rJHM/N
\text{A59})\text{JHM} slightly increased survival time compared to infection with rJHM (Fig. 2-2B). It is difficult to measure differences in virulence between these two highly neurovirulent viruses. Attenuation conferred by expression of A59 N in the JHM background was more easily observed in a comparison of rJHM/S
\text{A59}/N
\text{A59} and the already attenuated rJHM/S
\text{A59} (Fig. 2-2D).

The differences in neurovirulence observed in N exchange viruses compared with wild-type isogenic viruses were reflected in the small but statistically significant differences in replication in the CNS after i.c. inoculation (Fig. 2-4). More strikingly, expression of JHM N within the A59 background conferred increased antigen expression in the brain while reciprocal expression of A59 N within the JHM background conferred a decrease in the level of antigen expression in the brain (Fig. 2-5A to D). In agreement with previous studies from our lab, the level of MHV antigen expression in the brain is a consistent correlate of virulence (14, 39).

We considered several possible mechanisms to explain the differences in neurovirulence and antigen spread in the brain between N exchange viruses. (i) Expression of JHM N could confer an ability to spread among neurons more efficiently or expand the tropism of the virus for more neuronal subtypes. (ii) JHM N expression could enhance spread among glial cells or between glial cells and neuronal cells; this seemed unlikely as no differences in spread were observed in primary astrocyte cultures (data not shown). (iii) JHM N could alter the immune response, allowing greater spread than that of parental
rA59 strain. The rA59 strain is cleared by a robust CD8 T-cell response (11, 20), and we observed previously that this property mapped within the 3' end of the genome but not to the JHM spike (19). This left the possibility that N may be responsible for differences in T-cell response.

In order to explore the mechanism by which expression of JHM N enhances the amount of antigen expression in the brain, we examined spread in primary hippocampal neuronal cultures. Previous studies of A59/JHM chimeric viruses demonstrated that expression of JHM S within the A59 background enhances *in vitro* neuron-to-neuron spread (39) but not to the extent of spread with rJHM (unpublished data). This suggested the possibility that another viral protein could play a role in neuron-to-neuron spread. It had previously been reported that JHM N is closely associated with microtubules in the infected rat neuronal cell line OBL-21 and that N shows strong homology to microtubule binding protein tau (37). This suggested that N could be playing an important role in trafficking of the virus, and we hypothesized that the JHM N may be optimized for fast axonal transport relative to A59 N. However, exchange of N genes failed to confer a measurable difference in spread among primary hippocampal neurons (Fig. 2-6A to D), implying that the mechanism by which JHM N enhances antigen distribution in the brain is not through enhanced neuron-to-neuron spread. While there are no data indicating that there are any qualitative differences in the types of neurons in the brain infected by JHM compared to A59, we have used only hippocampus-derived neurons, and it is possible that JHM infects a wider range of types of neurons than A59.

To determine if the difference in the ability to induce a robust T-cell response in the CNS maps to the N gene, we compared the T-cell response of N exchange viruses with the isogenic parental viruses rA59 and rA59/S_{JHM}, which induce robust virus-specific T-cell responses, and with rJHM, which induces a very minimal response. Infection with rJHM/N_{A59} resulted in an increased T-cell response in the brain relative to isogenic rJHM,
suggesting that JHM nucleocapsid expression is required for the minimal T-cell response phenotype of JHM (Fig. 2-7A). There was a statistically significant difference in both CD8 and CD4 T-cell numbers. The resulting T-cell response, however, was consistently lower than that of rA59/S_{JHM}, suggesting that although JHM N contributes to the weak T-cell response phenotype, other proteins in the JHM background are likely to be important as well (Fig. 2-7A). Exchange of N genes in the A59 background viruses showed a reciprocal effect. Infection with rA59/S_{JHM}N_{JHM} resulted in a small decrease in the induced T-cell response compared to infection with isogenic rA59/S_{JHM} and an increase over the response to rJHM (Fig. 2-7B); similarly, the total CD8 T-cell response induced by rA59/N_{JHM} was statistically significantly less than that induced by rA59 infection (Fig. 2-7C). Thus, while the genotype of N appears to have a modest effect on the T-cell response to MHV infection, it is unlikely that this is the primary mechanism by which JHM N confers increased virulence when expressed in the A59 background.

Differences in the type I IFN response could also play a role in the virulence associated with JHM N. A59 N was shown to serve as a type I IFN antagonist when expressed from a recombinant ΔE3L vaccinia virus (49), and infection of type I IFN receptor-deficient mice (IFNAR^{-/-}) demonstrated that type I IFN is crucial for defense during early infection with A59 and JHM in vivo (4, 15, 42). Thus, it is possible that JHM N could be a more potent IFN antagonist than A59 N and thus compromise host defenses. However, this is unlikely, as supported by the following observations: rA59 and rJHM induced similar levels of type I IFN in the brains of infected mice, and both strains were significantly more lethal in IFNAR^{-/-} mice than in wild-type isogenic mice (42). In the absence of a type I IFN response, rJHM is able to spread from the brain into the periphery and replicate in the liver as well as in other organs not usually infected by JHM (15, 42), underscoring the inability of JHM to overcome the antiviral state induced by IFN in wild-type mice. Furthermore, there were no differences in the ability of A59 and JHM to induce type I IFN
in fibroblasts or in macrophages or to replicate in L2 cells (43) or macrophages (unpublished) that were pretreated with IFN-β.

In addition to the differences in neurovirulence, A59 and JHM vary greatly in their ability to replicate in the liver and cause hepatitis. The ability of A59 to replicate to high titers in the liver and cause severe hepatitis does not map to the S gene but, rather, to one or more other genes in the 3’ end of the A59 genome (31). The studies presented here clearly show that the ability to induce hepatitis in C57BL/6 mice, in the context of A59/JHM chimeras, does not depend on the N gene of the A59 strain and that expression of the N and S genes of A59 within the JHM background is not sufficient to induce hepatitis (Fig. 2-8B). Though replacing the A59 N gene with that of JHM did not prevent the virus from causing severe hepatitis, expressing JHM N in the A59 background did significantly reduce viral liver titers by about 10-fold at 5 days postinfection (Fig. 2-8A) and caused slight, though insignificant, decreases in hepatitis and viral antigen expression in the liver (Fig. 2-9A to C). Thus, although expression of JHM N clearly does not block MHV-induced hepatitis, expression of JHM N may play a minor role in decreasing the level of replication in the liver. Levy and colleagues demonstrated that the highly hepatotropic strains MHV-3 and A59 (but not the nonhepatotropic JHM strain) induce fibrinogen-like protein 2 (fgl2) and that fgl2 induction is responsible for the induction of fulminant hepatitis in MHV-3-infected mice (6, 33). Interestingly, the induction of fgl2 was mapped to the N protein of hepatotropic strains (33). However, in the studies presented here, replacement of the N gene of A59 with that of the nonhepatotropic JHM did not prevent the resulting virus (rA59/NJHM) from causing severe hepatitis. This demonstrates that in the context of A59/JHM chimeras, the induction of hepatitis does not depend on the N gene of the hepatotropic A59.

It is important to note that the A59 N gene contains within it another gene, in the +1 reading frame, encoding the internal, or I, protein. The I protein is a mostly hydrophobic
23-kDa structural protein of unknown function (9). Interestingly while I protein is expressed by most MHV strains, the JHM strain does not encode the I protein (9, 36). Thus, the I gene could possibly be responsible for one or more of the properties attributed to N including the pathogenic differences between A59 and JHM. However, the data presented here demonstrate that expression of the I gene from within the JHM genome (rJHM/N_{A59}) is not sufficient to confer the ability to induce hepatitis. Furthermore, the I gene is unlikely to be an important determinant of pathogenesis as an internal gene knockout recombinant A59 replicated similarly to an isogenic wild-type virus in vivo, and no major differences in clinical signs were reported (9).

The data presented here demonstrate that the N gene plays a major role in the extent of neurovirulence and a minor role, if any, in MHV-induced hepatitis. While we have not defined the mechanisms by which the N gene influences pathogenic outcome, the data do, however, suggest that increased neurovirulence associated with expression of the JHM N gene within the A59 background is not a result of enhanced neuron-to-neuron spread. The data furthermore indicate that there may be an influence of N on the induction of a robust T-cell response, which is crucial to virus clearance in the CNS. Future studies will be directed at further understanding the differences in virus-host interactions between chimeric and isogenic wild-type viruses. This will focus on the cytokines elicited in response to MHV and how the spread of virus within the CNS affects the host immune response.

ACKNOWLEDGMENTS

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Chapter Three

ABROGATION OF INTERNAL PROTEIN EXPRESSION BY THE MURINE CORONAVIRUS STRAIN A59 HAS NO AFFECT ON MORTALITY AFTER INTRACRANIAL AND INTRAHEPATIC INOCULATION
ABSTRACT

Most strains of the murine coronavirus mouse hepatitis virus (MHV) contain an additional open reading frame within the nucleocapsid gene that encodes the internal (I) protein. The I protein is a 23kDa hydrophobic viral membrane associated protein of unknown function. As demonstrated in Chapter 2, characterization of recombinant viruses in which the nucleocapsid genes, and the I genes within, were exchanged between the hepatovirulent, weakly neurovirulent A59 strain and the nonhepatotropic highly neurovirulent JHM strain demonstrated that expression of the JHM nucleocapsid enhances neurovirulence, while expression of the A59 nucleocapsid is attenuating. Since the A59 strain expresses I protein and the JHM strain does not, it was possible that the observed differences in virulence conferred by exchanges of the N gene were due to differences in the I gene rather than in N. Here we demonstrate a recombinant A59 in which expression of the I gene has been abrogated, display similar virulence to wild type A59 after intracranial and intrahepatic inoculation. Thus differences in virulence between nucleocapsid exchange viruses are most likely due to differences in nucleocapsid protein and not differences in expression of the I protein.

RESULTS and DISCUSSION

The murine coronavirus mouse hepatitis virus (MHV) is a large enveloped single-stranded and positive-sense RNA virus (5). MHV infection provides models of acute encephalitis, chronic demyelinating diseases such as multiple sclerosis, viral hepatitis, and severe acute respiratory syndrome (15)(1)(3). Two strains in particular, rJHM and rA59, have been extensively studied. rJHM causes severe encephalitis and is not hepatotropic (4)(11), while rA59 causes more mild encephalitis and moderate hepatitis
rJHM induced encephalitis is almost uniformly fatal at doses of only a few PFU, while the LD$_{50}$ of rA59 is approximately 3 x $10^3$ to 5 x $10^3$ PFU after IC inoculation (6)(8).

A59/JHM chimeric viruses have been used to map the viral determinants of pathogenesis. Chimeric viruses in which the first two-thirds of the genome were exchanged mapped major virulence determinants to the 3’ third of the genome (9). Expression of the JHM spike within the A59 background (rA59/S$_{JHM}$) conferred a reduced LD$_{50}$ of less than 10 PFU and enhanced spread in the CNS (6)(13)(8), while a chimeric virus expressing A59 spike in the JHM background (rJHM/S$_{A59}$) displayed increased LD$_{50}$ and decreased spread in the CNS relative to rJHM (10)(6)(2). However, rA59/S$_{JHM}$ was not as virulent as rJHM, suggesting that there are virulence factors other than spike (6)(13)(8)(14).

One such virulence factor is the nucleocapsid protein (N). A virus expressing the JHM N in the rA59 background (rA59/N$_{JHM}$) displays an LD$_{50}$ of less than 10PFU and both replicates to a higher titer and spreads more in the CNS than rA59 (2). The reciprocal chimeric virus, expressing A59 N from within the JHM background (rJHM/N$_{A59}$), replicated to a lower titer and spread less in the CNS than rJHM, killing mice slightly, but significantly, slower than rJHM (2). In addition rJHM/N$_{A59}$ was not able to replicate in the liver, indicating that expression of A59 N is not sufficient to not confer hepatotropism (2). Infection with the complementary chimeric recombinant, rA59/N$_{JHM}$ demonstrated no significant differences compared to rA59 in degree of hepatitis and spread of viral antigen in the liver (2). However, rA59/N$_{JHM}$ did replicate to slightly, but significantly lower titers than rA59, and while not statistically significant, hepatitis and viral antigen in the liver was reduced as well (2).

The I protein is encoded within the +1 reading frame of the N gene. I protein is a 23kDa hydrophobic viral membrane associated protein of unknown function (4). Paul Masters
and colleagues selected a recombinant A59 virus, in which expression of the I gene is abrogated by mutations that are silent for N, but mutate the initiation codon and create a premature stop codon in aa 4 of the I gene (Alb110). A wild type isogenic control (Alb111) was also selected. The Weiss lab collaborated with Dr. Masters to show that there were no major differences between Alb110 and Alb111 replication in vitro or in the brain or liver. However, Alb110 has a small plaque phenotype, which suggests that ablation of the I gene might have some significance to infection. Additionally it was noted that there may have been a small reduction in replication of Alb110 in the liver, although the statistical significance could not be determined since the number of infected animals was too small (4).

Since the I gene is located within the nucleocapsid gene, exchange of the nucleocapsid genes also included exchange of the I genes. Unlike rA59 and most other MHV strains examined, rJHM does not express I (4, 12). This is due to a premature stop codon at amino acid 18. The pathogenic analysis of N exchange viruses is complicated by these differences in I expression. Therefore, we addressed two questions: 1) Does I protein expression play a role in neurovirulence or hepatovirulence? 2) What impact, if any, did I protein have on the differences observed between N exchange viruses and their isogenic controls?

**I gene expression does not influence MHV CNS pathogenesis.** To determine the affect of I on neurovirulence, the I gene mutant virus, Alb110, was compared to its wild type control, Alb111. rA59 was included as a second wild type control because Alb111 was more virulent than expected in preliminary experiments. Viruses were serially diluted with PBS containing 0.75% bovine serum albumin and five mice each were inoculated intracranially with $10^4$ PFU, $10^3$ PFU, and $10^2$ PFU. The kinetics of survival of Alb110 and Alb111 infected mice were not significantly different at any dose (fig. 3-1A-C). At all three doses rA59 was attenuated compared to Alb111, but the difference was only significant
at the dose of $10^3$ PFU ($p=0.044$, Fig. 3-1B). This resulted in an LD$_{50}$ of 2400 PFU for rA59, while Alb110 and Alb111 both had LD$_{50}$s below 100 PFU. These results imply that I is not an important virulence determinant in the CNS, but also imply that Alb111 is much more virulent than rA59.

**I gene expression does not significantly influence hepatovirulence.**

To determine the affect of I gene expression on hepatovirulence we compared two sets of viruses differing in I gene expression following intrahepatic inoculation. These are Alb110 vs. Alb111 and rA59 vs. rA59/N$_{JHM}$ Five mice each were inoculated intrahepatically with 100 PFU of virus. There were no significant differences in mortality between mice infected with Alb110 and Alb111. For both viruses infected mice succumbed to infection by day 8 post infection (Fig. 3-2). The difference between rA59 and rA59/N$_{JHM}$ was not significant, but was very close ($p=0.054$, Fig. 3-2). The near significant decrease in hepatovirulence of rA59/N$_{JHM}$ compared to rA59 is consistent with our finding that rA59/N$_{JHM}$ replicated to slightly, but significantly, lower titers in the liver than rA59 (2), and that rA59/N$_{JHM}$, while not significant, had trends of reduced viral antigen distribution in the liver and reduced hepatitis compared to rA59 (2).

Both Alb110 and Alb111 were significantly ($p=0.044$) more virulent than our lab wt rA59 after intracranial inoculation. Though it is unclear how the enhanced virulence of this background may have influenced our results, it would be more relevant to study the role of internal protein in the background of our lab rA59. The enhanced virulence of Alb110 and Alb111 is a potentially interesting discovery. The Alb111 genome has not been sequenced, but it was derived form similar virus and plasmid to our lab rA59 and should bare very few sequences differences. Thus sequence comparison of the Alb111 and rA59 genomes could lead to greater insight of virulence determinants.
Figure 3-1: Survival curves of intracranially infected 4 week old C57BL/6 mice. Five mice each were inoculated with (A) 10^2 PFU, (B) 10^3 PFU, and (C) 10^4 PFU of Alb110 (I'), Alb111 (I'), and rA59. Mice were observed for 21 days for mortality. Survival of Alb110 and Alb111 infected mice are not significantly different while survival of rA59 and Alb111 infected mice are significantly different at a dose of 10^3 PFU (p= 0.044, Gehan-Breslow-Wilcoxon test).
Figure 3-2: Survival curves of intrahepatically infected 4 week old C57BL/6 mice. Five mice each were inoculated with $10^2$ PFU of Alb110 (I'), Alb111 (I'), rA59, and rA59/N_{JHM}. Mice were observed for 21 days for mortality. Survival of Alb110, Alb111, and rA59 infected mice are not significantly different, while survival of rA59 and rA59/N_{JHM} infected mice displayed a difference that was near to significant ($p=0.054$, Gehan-Breslow-Wilcoxon test).

The fact that no significant virulence differences were observed between Alb110 and Alb111 after intracranial or intrahepatic inoculation suggests that the I is not a significant virulence factor. Nevertheless, the fact that I gene expression is maintained by most MHV strains suggests it provides an advantage at least under certain conditions, although it is unlikely to be important in CNS infections as the most neurovirulence JHM does not express I. The observation that Alb110 produces smaller plaques than Alb111 suggests it may confer some advantage in replication in vitro (4). Perhaps I provides an advantage only under certain routes of infection or in organ systems such as the gastrointestinal track not investigated here.

Since differences in I protein expression did not have a discernable impact on CNS or liver pathogenesis, the N protein is most likely responsible for the differences in virulence observed when the N genes were exchanged between A59 and JHM.
REFERENCES

Chapter Four

MUTATIONS IN THE HEMAGGLUTININ-ESTERASE PROTEIN THAT ABROGATE EXPRESSION IN A JHM INFECTIOUS CLONE SYSTEM HAVE NO AFFECT ON NEUROVIRULENCE
ABSTRACT

The study of the pathogenesis of the neurovirulent murine coronavirus strain JHM was limited by the lack of a convenient reverse genetics system to manipulate genes beyond the 3’ third of the genome. Here we describe a reverse genetics system in which the JHM genome was amplified into seven cDNA fragments, each of which was cloned separately into a bacterial plasmid. These individual subgenomic cDNA clones allow for easy manipulation of the genome using standard molecular biology techniques. The fragments can be assembled into a full genomic-length cDNA to be used as a template for the transcription of an infectious genome RNA, which can then be transfected into cells to generate infectious virus. This technique was used to generate isogenic JHM clones that differ only in expression of the hemagglutinin-esterase (HE), an accessory structural protein that has sialic acid binding and acetyesterase activity. We were not able to detect differences in neurovirulence between wild type JHM (HE+) and a mutant unable to express the HE protein (HE-), suggesting that HE is of minimal importance during CNS infection by JHM.

INTRODUCTION

Coronaviruses are large, enveloped, single-stranded and positive-sense RNA viruses within the nidovirus superfamily (28). The murine coronavirus, mouse hepatitis virus (MHV) is a collection of strains that cause neurological, hepatic, enteric, and respiratory disease (6, 69). The nature and outcomes of these diseases are dependent on the viral strain and route of infection (69). MHV serves as a model for studying both acute and chronic neurological diseases including encephalitis and chronic demyelinating diseases such as multiple sclerosis (6). MHV also serves as a model for studying virus-induced
hepatitis and viral respiratory infections, such as severe acute respiratory syndrome (SARS; 17).

The study of MHV pathogenesis is greatly enhanced by the availability of three types of reverse genetic systems, targeted recombination, use of a vaccinia virus vector and the use of full genome length cDNA. Much of the murine coronavirus pathogenesis research has been carried out using targeted recombination systems (32, 37, 39, 48). However, even the most advanced version of the targeted recombination system limits genetic changes to the 3’ one third of the genome, that is the spike gene through the 3’ end of the genome (32, 39). This system relies on recombination between a synthetic RNA transcribed from a plasmid containing the 3’ end of the genome and a chimeric MHV in which the spike gene has been replaced with that from the feline infectious peritonitis virus (FIPV). The selection of recombinants depends on the specificity of the spike coronavirus proteins. Recombination occurs in Felis catus whole fetal (FCWF) cells infected with the chimeric MHV and transfected with synthetic RNA. Recombinant virions that regain the MHV spike, which also gain downstream changes from the transfected RNA, are selected for by their ability to infect 17Cl1 mouse fibroblast cells. This system has been used widely to select mutants and chimeric viruses for the strains A59, JHM and MHV-1 (32, 39, 50, Leibowitz et al., in press). However there are imitations of this technology. Firstly, the 5’ two thirds of the genome, including the 20 kb replicase gene, ORF2a, and the HE gene cannot easily be manipulated with this approach. This system was modified to allow the manipulation of the ORF2a and HE genes within the A59 background. This was done by extending the plasmid to start at residue 20497, which is 5’ to ORF2a and HE, and engineering a recipient virus in which the ORF2a and HE genes were deleted (43). A second drawback to the targeted recombination technology is the possibility of additional crossover events. The selection of a recipient virus in which the 3’ gene order had been rearranged (19) was carried out to eliminate this possibility.
Reverse genetics systems utilizing full length infectious clones have been used for several coronaviruses including the A59 strain of MHV (76). Using this approach the A59 genome was reverse transcribed into seven fragments and cloned into bacterial plasmids. These fragments were flanked by either convenient naturally occurring restriction sites or BsmBI (Esp3I). The BsmBI restriction enzyme cuts away from its recognition site allowing for the removal of the site and a seamless ligation of fragments; this is called the "no see’em" technique. Desired mutations or gene substitutions can be made by standard site directed mutagenesis and cloning techniques in any of the seven plasmids. A ligated full length cDNA clone can then be used as a template for in vitro RNA transcription. The full length genomic RNA transcript is then transfected into baby hamster kidney cells stably expressing the MHV receptor, CEACAM1a (BHK-MHVR cells). In addition transfection of in vitro transcribed nucleocapsid mRNA is also transfected because it was observed to enhance viral replication (5, 76). Similar approaches have also been used to clone the SARS coronavirus (75) and transmissible gastroenteritis virus (TGEV; 74). HCoV-229E and Infectious bronchitis virus (IBV) were also cloned into bacterial plasmids, but these were then used to further engineer the viruses into vaccina virus (12, 65). TGEV bacterial plasmids have also been further engineered into an artificial bacterial chromosome (3). We have constructed a full length JHM cDNA clone and used it to engineer mutations that abolish expression of the MHV hemagglutinin-esterase (HE) gene.

HE is an accessory protein expressed by some group II coronaviruses, including many, but not all MHV strains. A59 for example, has an HE pseudogene and therefore does not express HE (59, 62, 72). HE is a class I membrane protein of about 65 kDa that forms disulfide-bonded homodimers, (11, 30-31, 35-36, 59-60), and these or multimers thereof form 5 to 7 nm projections on the surface of virions (10, 64). HE binds to O-acetylated sialic acid residues and also has acetylesterase activity, allowing it to function as a sialic acid receptor destroying enzyme. This presumably allows for reversible attachment to
cells. Though HE has sialic acid binding activity, cell entry for MHV clearly relies on the spike protein (26, 46). Spike binds members of the mouse carcinoembryonic antigen-related glycoprotein family (e.g. CEACAM), which are the only known MHV receptors (23, 25, 27, 70), and mediates viral-cell or cell-to-cell membrane fusion. For human coronavirus OC43 (HCoV-OC43) and bovine coronavirus (BCoV) the spike protein, not HE, is primarily responsible for attachment to sialic acid residues (38, 57-58, 63, 71), but for MHV, HE is primarily responsible for sialic acid binding (41). Depending on the MHV strain there are two possible specificities of HE binding and acetylesterase activities. The HESs of some strains, such as MHV-S and JHM, bind and cleave 5-N-acetyl-4-O-acetyl neuraminic acid (Neu4,5Ac₂) (55, 71), while the HESs of other strains, such as DVIM, bind and cleave 5-N-acetyl-9-O-acetyl neuraminic acid (Neu5,9Ac₂) (18, 61).

The role of hemagglutinin esterase was examined by restoring HE expression in rA59, a virus that normally does not express HE. To do this the HE pseudogene of the A59 strain was replaced with the MHV-S HE gene, either wild type (rMHV-A59S-HE⁺), or mutant HE in which acetylesterase activity was abrogated (rMHV-A59S-HE°), or mutant HE in which protein expression was abrogated (rMHV-A59S-HE-) (34, 43). In this study MHV-S HE expression appeared to have no effect on replication in the CNS or the liver. Furthermore, in vitro passaging of both rMHV-A59S-HE⁺ and rMHV-A59S-HE° selected for mutations that prevented HE insertion into the membrane. Another set of chimeric viruses were selected that are isogenic to the first set except that they express JHM spike in place of A59 spike; they are named rMHV-JHMS-HE⁺, rMHV-JHMS-HE° and rMHV-JHMS-HE- (34). In this context HE expression increased neurovirulence as well as viral spread in the brain. This suggested that cooperation between the spike and HE proteins might be necessary for HE to enhance virulence or that JHM spike might direct virus to cell types in which HE can enhance attachment and subsequent entry. Interestingly, esterase activity was not needed for enhanced neurovirulence, suggesting that binding activity, not enzymatic activity, is responsible for enhanced virulence (34).
These experiments suggested that the expression of HE by the JHM strain may contribute to the high neurovirulence and spread observed during JHM infection of the CNS. The HE of JHM is 96% identical to the MHV-S HE, and both have similar binding activity to Neu4,5Ac₂ and similar enzymatic activity when incubated with fluorescein diacetate, 4-methylumbelliferyl acetate, and p-nitrophenyl acetate (55, 71). However, it could not be concluded from these experiments that JHM HE plays an important role in JHM neurovirulence because the HE used in these studies was derived from MHV-S and not JHM and the chimeric viruses used in this study had A59 background genes, not JHM, and this can significantly alter tropism and pathogenesis (15, 33, 49, 52, 53). Thus, to better address the role of HE in JHM CNS disease, we sought to select isogenic JHM viruses differing only in HE expression. Targeted recombination, for the reasons stated above, was not ideal for the generation of these viruses, so we constructed a full length cDNA clone of the JHM genome and used it to generate isogenic HE⁺ and HE⁻ viruses (icJHM; icJHM/HE⁻).

MATERIALS AND METHODS

Cells and Viruses. Murine fibroblast (L2 and 17Cl1) cells were maintained in Dulbecco’s minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% amphotericin B (Fungizone), 10mM HEPES, and 2 mM L-glutamine. BHK-MHVR cells were maintained in the same medium with the addition of 600µg/mL geneticin (71-75% pure, GIBCO) for maintenance of CEACAM1a expression.

Recombinant JHM was selected using a targeted recombination system (49). icJHM and icJHM/HE⁻ were generated with the infectious clone system described in this paper. Previously described viruses that were used include: rA59 (52), MHV-4 (the wild type
virus from which rJHM was derived (16, 51), rMHV-A59S-HE⁺ (rA59 expressing the HE of MHV-S (43), and rMHV-JHMS-HE⁺ (rA59 expressing the HE of MHVS and the JHM spike (34).

Construction of JHM cDNA clones. The same rJHM stock that was sequenced (GenBank: FJ647219.1) was used to infect L2 cells. RNA was extracted using QiaShreeder columns (Qiagen) and the RNeasy RNA extraction kit (Qiagen) according to kit instructions. Reverse transcription was performed using SuperScript II (Invitrogen) and random hexamer primers. The reaction was carried out at 50°C for 1h followed by 94°C for 2 minutes. cDNA was amplified by PCR with Expand Long TAQ polymerase (Roche) to create seven fragments (A-G; see Table 4-1 for primers used). The reaction conditions were: 2 minutes of denaturing at 94°C followed by 25 cycles of 94°C for 30 seconds, 58°C for 30 seconds, and 68°C for 1 to 7 minutes (depending on amplicon length). PCR products were cloned into pCR2.1 TOPO TA (Invitrogen), pCR4 TOPO TA (Invitrogen), or pcrSMART vectors (Lucigen, Middleton WI). Multiple clones for each fragment were sequenced and mutations were corrected by either exchanging sections between clones of the same fragment or through site directed mutagenesis. The F fragment was generated as four separate fragments that were ligated together. The last fragment (G) was cloned into pJHM, a plasmid used for targeted recombination that contains the 3’ one third of the JHM genome, thereby extending the 5’ end 1139 nucleotides.

Generation of icJHM/HE⁺. To generate an icJHM in which the expression of HE has been ablated, a section of the icA59 F fragment was amplified and cloned into the icJHM F fragment. The amplicon spans the last 48 codons of ORF2a to codon 46 of HE. The amplified region of A59 includes a single amino acid difference in ORF2a, which was corrected in the final construct, a mutation in the transcriptional regulatory region of HE reported to prevent transcription (UAAUAAAAC to UAAUAAGC) (59, 72), an AUG to AAG
mutation of the start codon of HE (though there is a potential alternative start codon
downstream), and a premature stop at codon 18 (as measured from the JHM start site).

Table 4-1: Primers used in the construction of infectious clone plasmids. Bold
sequences represent restriction sites that were added (icJHM A Forward has an added
Pmel site) and the rest are BsmBI sites. The underlined sequence represents a T7
promoter

**Assembly of cDNA clones.** icJHM plasmids A through G were restriction digested,
electrophoresed on a 0.8% agarose gel and stained with ethidium bromide. A darkreader
lightbox (Clare Chemical) was used to visualize the bands, which were then purified

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<th>Primer Name</th>
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<td>icJHM A Reverse</td>
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<tr>
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<tr>
<td>icJHM B Reverse</td>
<td>5’-ACTGAACTCGCGTTAAGGC-3’</td>
<td>icJHM B/C junction</td>
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</table>
using the QIAquick gel extraction kit (Qiagen). Equal molar ratios of each fragment were ligated (about 2 µg of DNA total) with T4 ligase at 4°C overnight. The ligated cDNA was phenol/chloroform extracted and precipitated.

**In vitro transcription and transfection of genomic transcripts.** In vitro transcription, driven by a T7 promoter present directly upstream of the cDNA, was carried out using TmMessage mMACHINE (Ambion), according to kit protocol with some modification (76). The nucleocapsid gene was amplified using primers encoding an SP6 promoter, and the TmMessage mMACHINE (Ambion) was used to make transcripts according to kit instructions. Both genomic and nucleocapsid RNA were added to 800ul of BHK-MHVR cells (~8x10^6 cells), and the cells were electroporated with three pulses of 850V at 25 µF from a Gene Pulser II electroporator (Bio-Rad). Electroporated BHK-MHVR cells were plated and virus was harvested when the cells showed signs of significant cytopathic effect (CPE). Viruses were plaque purified twice. Two plaques from the same transfection were used in further analyses.

**qRT-PCR of HE transcripts.** L2 cells were infected with virus at an MOI of 0.05. Twenty-four hours post infection, RNA was harvested using Qiashredder columns (Qiagen) and an RNeasy kit (Qiagen). DNase treated RNA (Turbo DNA-free kit, Ambion) was reverse transcribed utilizing Superscript III (Invitrogen) and random primers (Invitrogen). Quantitative PCR was performed with an iQ5 iCycler (Bio-Rad) using iQ SYBR Green PCR mix (Bio-Rad).

**Virus Concentration and Purification.** Two T175 flasks of 17Cl1 cells were infected per virus. Low pH medium (DMEM supplemented with 12% FBS, 1% amphotericin B (Fungizone), 10mM HEPES pH 6.5, and 2 mM L-glutamine, 5% tryptose phosphate broth, 8% sodium bicarbonate, 0.08% NaCl.) was used while virus was grown. When most cells showed CPE, cells were subjected to three cycles of freeze/thawing. Cells and
supernatant were centrifuged at high speed to clarify. Clarified supernatant was spun through 20% sucrose/STE (0.01M Tris pH 7.4, 0.1M NaCl, 0.001M EDTA) for 2 hours at 25000 rpm in a SW28 rotor. Virus pellets were resuspended in a total of 500 µl of PBS for each virus.

**Western blot analysis for HE protein.** Concentrated virus preparations were boiled in Laemmli buffer without β-mercaptoethanol for 10 minutes. Samples were loaded onto a 7.5% non-reducing SDS-PAGE gel and following electrophoresis, electrotransferred to a PVDF membrane (Immobilon-P, Millipore). The membrane was blocked in Tris-buffered saline tween-20 buffer (TBS-T) containing 5% powdered milk for 30 minutes at room temperature, washed twice in TBS-T, and incubated with an anti-HE monoclonal antibody (Ab-823, kindly provided by Stuart Siddell) diluted 1:100 dilution in TBS-T at 4°C overnight. The membrane was washed twice with TBS-T and incubated with a horse-radish peroxidase conjugated anti-mouse secondary antibody (ImmunoPure Goat Anti-Mouse IgG, Thermo Scientific Lot # JB118235) at a 1:5000 dilution in TBS-T for 1 hour at room temperature. The ECL Plus Western Blotting Detection System kit (GE Healthcare) was used to develop the blot.

**Esterase Activity Assay.** Concentrated viral samples were run on a non-reducing SDS-PAGE gel as described above. Esterase activity was measured in the gel using the 91A kit (Sigma), with a modified protocol. Briefly: the gel was washed 3 times for 20 minutes in PBS with 1% Triton X, washed in PBS for 5 minutes, fixed for 30 seconds in a solution of 5 mL citrate solution, 13 mL acetone, and 1.6 mL 37% formaldehyde, and washed in distilled water. Then 0.65mL of sodium nitrite solution was added to 0.65 mL of Fast Blue BB Base and incubated in the dark for 2 minutes. This was added to 26 mL of water prewarmed to 37°C. 3.25 mL of Trizmal 7.6 buffer and 0.65 mL of α-naphthyl acetate solution were then added. The solution was mixed and the gel was incubated for 1 to 2 hours at 37°C and then washed in distilled water.
**Virulence Assays.** Viruses were diluted with PBS containing 0.75% bovine serum albumin. Five, four week old, C57BL/6 mice per dose were anesthetized with isoflurane and inoculated either in the left cerebral hemisphere or intranasally. Mice were monitored daily for 21 days and euthanized when they became moribund; euthanized mice were counted along with mice that died the following day.

**RESULTS**

**JHM reverse genetics system: construction of an infectious genome length cDNA clone.**

cDNA was transcribed from RNA extracted from rJHM infected cells using reverse transcriptase and random hexamer primers. This cDNA was used to amplify seven fragments by PCR and each was separately cloned into a bacterial plasmid (Table 4-1). The seven clones (A-G) correspond in genome position to the seven fragments used in the A59 infectious clone reverse genetics system described by Ralf Baric and colleagues (76) (fig. 4-1). The forward primer for the A fragment contained a T7 promoter, which was used to generate RNA transcripts. BsmBI (Esp3I) restriction sites were added to the primer ends forming A/B, C/D, D/E, E/F, F/G junctions; in the case of the B/C junction naturally occurring BbsI and BglI sites were available for ligation so BsmBI sites were not added. The BsmBI restriction enzyme is a type II restriction enzyme that directs cleavage downstream of the recognition site. When BsmBI sites are placed in the correct orientation, digestion cleaves off the restriction site and leaves unique sticky ends. Table 4-2 lists the fragment junctions. The JHM genome contains eight endogenous BsmBI sites. In order to use BsmBI for assembly, the endogenous sites were inactivated using site directed mutagenesis to create silent mutations. Other than these eight silent mutations, the cloned fragments are identical in sequence to that of rJHM (Genbank:
Coronaviruses contain sequences that can be unstable and/or toxic in bacterial plasmids \((8, 21-22, 29, 44-45)\) and toxicity appears to be due to expression since these sequences are more stable in vectors that tightly regulate expression and when the bacteria are grown at \(30^\circ C\) \((76)\). The positions of fragment junctions were empirically determined for A59 to disrupt sequences that were toxic and/or unstable in bacteria \((76)\). Since A59 and JHM are 94% identical, the genome was divided into fragments in the same fashion, assuming the toxic regions would be similar. In addition pcrSMART vectors where used for two of the most toxic fragments (C and D), because these vectors are designed to prevent leaky transcription. The more stable fragments were cloned into pCR2.1 or pCR4 TOPO TA vectors for convenience. The large size (6985 nt) of the F fragment made it difficult to clone as one piece; thus it was initially cloned as four smaller fragments, which were then ligated to form the larger F plasmid. Several clones of each fragment were sequenced and regions with mutations were exchanged for those with wt sequences. Other mutations were fixed utilizing site directed mutagenesis.

![Diagram](image)

**Figure 4-1: Schematic representation of the JHM genome.** The lengths and positions of the cloned JHM fragments A-G are shown relative to the JHM genome. Vertical lines represent the boundaries of individual fragments.

To assemble full genome-length cDNA, plasmids were digested and fragments were purified on agarose gels. Ethidium bromide present in the gel and a darkreader lightbox were used for the visualization. The darkreader utilizes blue light instead of UV to
fluoresce the ethidium bromide, which prevents UV damage to the DNA. Equal molar ratios of each fragment were ligated in a single reaction with T4 ligase. Ligated DNA was phenol/chloroform extracted and transfected into baby hamster kidney cells expressing the MHV receptor, CEACAM1a (BHK-MHVR). BHK-MHVR cells were placed in tissue culture flasks and when CPE developed supernatant containing virus was isolated and recombinant viruses were plaque purified twice. Viruses are referred to as infectious clone(ic) JHM.

Construction of isogenic recombinant icJHM except for HE expression (icJHM and icJHM/HE-).

In order to generate a virus that does not express HE, we ablated transcription of mRNA2-1 which is the mRNA that encodes HE, by inserting a portion of the icA59 HE pseudogene in place of that of icJHM HE within fragment F of the JHM clones. This fragment spans the last 48 codons of ORF2a to codon 46 of HE. There are three important sequence differences between rA59 and rJHM in this portion of the HE gene and these together ensure that the mRNA and protein will not be expressed and there should be little chance of reversion (fig. 4-2). These are 1) a mutation in the HE transcription regulatory sequence (TRS) from UAAUAAAC to UAAUAAGC, 2) a mutation of the AUG start codon of JHM HE to AAG, although another AUG is present shortly downstream, 3) and a premature stop mutation at codon 18 (relative to the JHM start codon). The TRS mutation was reported to prevent transcription of mRNA2-1 (59, 72), and the premature stop should prevent protein translation. The infectious clone system was used to generate wild type JHM (icJHM) and mutants unable to express HE (icJHM/HE-). The HE genes of plaque purified viruses were sequenced to confirm that they were correct.
Infectious clone JHM has similar levels of HE mRNA transcripts as recombinant JHM and MHV-4

In order to confirm that the wild type infectious clone expressed mRNA2-1 and to verify that icJHM/HE- did not express HE mRNA transcripts, we compared expression levels of mRNA2-1 with that of various relevant viruses. These included: 1) rMHV-JHMS-HE+, expressing the HE protein of MHV-S as described above (34), 2) MHV-4, which is the wild type non-recombinant JHM.SD virus on which recombinant JHM (rJHM) is based, 3) rA59, from which the pseudo-HE fragment was cloned for icJHM/HE-, 4) wild type icJHM, and 5) icJHM/HE-.

L2 cells were infected, and RNA was harvested and used to quantify both genomic RNA and HE transcripts using qRT-PCR. HE mRNA levels were normalized to genome levels and the mRNA2-1/genome RNA ratio for rJHM was set to 1, for comparison with other viruses. All wild type JHM viruses (icJHM, rJHM, and MHV-4) expressed a similar number of transcripts per genome (fig. 4-3). As expected icJHM/HE- did not express mRNA2-1 (fig. 4-3). rMHV-JHMS-HE+ expressed between 100 and 200 fold higher levels of transcripts per genome (fig. 4-3). This is consistent with previous reports that MHV-S expresses higher levels of HE mRNA than JHM (40, 72). Surprisingly and contradictory to earlier reports (59, 72)Liebowitz in press), rA59 also expressed mRNA2-1 in this assay; this suggests that our assay is more sensitive. To ensure that the signal detected for rA59 was indeed due to HE transcription it was confirmed through sequencing. Though rA59 may make transcripts, the pseudogene encodes a premature stop codon, so full length HE protein cannot be made.
<table>
<thead>
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<th>icJHM fragment</th>
<th>Restriction Site Junction</th>
<th>Location</th>
<th>Junction Overhang</th>
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<tr>
<td>A</td>
<td>5'-TTGTCAT'AAATAGACGNNNN-3'</td>
<td>3'-end of A BsmBI, nt 4885</td>
<td>A/B TTTA</td>
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<td></td>
<td>3'-AACACTTAATTACTCTGCNNNN-5'</td>
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</tr>
<tr>
<td>B</td>
<td>5'-NNNNCGTCTCA'TAAATTTGAT-3'</td>
<td>5'-end of B BsmBI, nt 4886</td>
<td>A/B TAAA</td>
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<td>3'-NNNNCGAGAGTATTT,AACATA-5'</td>
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<tr>
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<td>5'-CTTTGCTC'TAACGGGAGGTTTC-3'</td>
<td>3'-end of B BglI, nt 9570</td>
<td>B/C TTA</td>
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<td></td>
<td>3'-GAAACGGAAATTGCCGCTCAAG-5'</td>
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<tr>
<td>C</td>
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<td>5'-end of C BglI, nt 9571</td>
<td>B/C TAA</td>
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<td>C/D GATT</td>
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<td>D</td>
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<td>C/D AATC</td>
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<td>E</td>
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<td>F</td>
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<td>5'-end of G BsmBI, nt 22755</td>
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<td>3'-NNNNCGAGACGGC, TGACATGG-5'</td>
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</table>

Table 4-2: Junctions of icJHM. Sequences in bold represent restriction sites, arrows indicate cleavage sites, and underlined sequences are the resulting unique overhangs, which are repeated in the far right column. Location shows the nt positions of the JHM genome that each fragment begins and ends with as well as a restriction site used.
Figure 4-2: Schematic representation of the icJHM and icA59 HE genes. The intergenic region is shown as a small box, while the coding region is represented as a large box. Gray represents icJHM sequence and white represents icA59 sequence. Important differences between icA59 HE and icJHM HE are highlighted (the spacing of these differences are exaggerated relative to the size of the gene). These include an A to G mutation in the TRS of icA59, an AUG to AGG mutation at the start site, an alternative start site at codon 4 (threonine to methonine) and a premature stop at codon 18. Below represents the HE gene as it has been engineered in icJHM/HE’, through cloning of the intergenic region and 5’ end of the icA59 HE into icJHM HE.

Figure 4-3: Quantification of HE mRNA. qRT-PCR was performed on RNA extracted from virus infected L2 cell to quantify HE mRNA as well as genome RNA. The ratio of HE transcripts to genome for rJHM was set to 1. Shown are the HE transcripts per genome relative to rJHM.
**IcJHM expresses HE protein with acetyesterase activity, while icJHM/HE⁻ does not.**

To confirm that icJHM expresses HE protein, icJHM, icJHM/HE⁻, rMHV-A59S-HE⁺, and rA59 were concentrated and partially purified by pelleting through 20% sucrose. Concentrated virus samples were electrophoresed in SDS-polyacrylamide gels under non-reducing conditions and transferred to a membrane. They were then stained with monoclonal anti-HE antibody (Ab-823, kindly provided by Stuart Siddell). HE protein was detectable in the two icJHM and in the rMHV-A59S-HE⁺ virus preparations (fig. 4-4A). HE bands appeared near the 132kDa marker, which is consistent with a homodimer of two 65kDa proteins. As expected, there was no detectable amount of HE protein in the two icJHM/HE⁻ or the rA59 samples (fig. 4-2A). Thus the two icJHM isolates express HE protein, but at a much lower level than rMHV-A59S-HE⁺. Not shown in this blot rMHV-JHMS-HE⁺ also expresses more protein than icJHM, which is consistent with the difference in mRNA levels (fig. 4-3).

Comparison of rMHV-JHMS-HE⁺ with esterase activity negative rMHV-JHMS-HE⁻ virus, demonstrated that the ability of HE proteins to enhance neurovirulence was not dependent on enzymatic activity (34). However, this did not prove that enzymatic activity would not be important if HE were expressed from a JHM background virus. Thus, we investigated whether icJHM expressed an enzymatically active protein. Thus an in-gel esterase activity assay was performed. In this assay enzymatically active HE hydrolyses a substrate and activates a dye, resulting in the staining of enzymatically active HE bands. Esterase activity was observed for both samples of icJHM and for rMHV-A59S-HE⁺ (fig. 4-4B). Esterase activity was not observed for icJHM/HE⁻ or rA59 (fig. 4-4B). Furthermore, the esterase activity was roughly proportional to the amount of protein detected by antibody (fig. 4-4).
Figure 4-4: HE protein expression and activity. Partially purified virus preparations were analyzed on a 7.5% SDS polyacrylamide gel under non-reducing conditions. (A) The gel was blotted onto a PVDF membrane and incubated with anti-HE mAb. rMHV-A59S-HE⁺ was applied at 1/10 dilution as a positive control. (B) The gel was incubated with substrate that when hydrolyzed activates a dye that stains esterase positive HE bands (see materials and methods). For both Westerns and esterase activity assays the HE protein migrated just below the 132kDa marker (consistent with homodimer formation), though the western blot shown was distorted causing rMHV-A59S-HE⁺ to appear to run higher.

No significant differences in neurovirulence were observed between the wild type icJHM and icJHM/HE⁻

We investigated whether HE expression conferred enhanced neurovirulence to icJHM as we had observed previously for rMHV-JHMS-HE⁺ and rMHV-JHMS-HE⁻ (34). Thus, weanling C57BL/6 (B6) mice were infected intracranially with 10 PFU of icJHM, icJHM/HE⁻ or rJHM. rJHM was included as a control to verify that icJHM behaved as wild type rJHM. For all three viruses all mice died by day 9 with similar kinetics and due to the extreme lethality of all viruses, LD$_{50}$ values could not be determined (fig. 4-5).

We then carried out infections by the intranasal route, which requires higher titers to induce disease and kills the mice with slower kinetics, making it easier to detect pathogenic differences among highly virulent viruses. We infected mice with 2000 PFU of icJHM, icJHM/HE⁻, or rJHM. As with intracranial inoculation the kinetics of infection was similar for all three viruses (fig. 4-5).

These experiments demonstrate that HE does not play an important enough role to affect the outcome of infection via the intracranial or intranasal route.
DISCUSSION

While expression of MHV-S HE in the context of rMHV-A59S-HE did not enhance neurovirulence (43), the enhanced spread and mortality of rMHV-JHM-HE+ demonstrates that MHV-S HE can enhance neurovirulence when expressed in conjunction with JHM S (34). The fact that MHV-S HE is similar to that of JHM in sequence, binding, and enzymatic activity (55, 71), and the observation that MHV-S HE enhances neurovirulence when paired with the JHM spike, and not A59 spike(34), led us to believe that the HE gene of JHM may, by a similar mechanism, enhance neurovirulence of JHM. Somewhat surprisingly, HE could not be demonstrated to play an important role in the neuropathogenesis of JHM during infections initiated by inoculations by either the intracranial or intranasal routes. One possible explanation for the difference is the 100-200 fold lower expression of HE observed with icJHM compared to rMHV-JHMS-HE+. Other possible contributing factors could be the rA59 vs. icJHM backgrounds or the MHV-S vs. JHM HE genes.

icJHM (and the other wild type JHM viruses rJHM and MHV-4) may not express enough HE protein to have an impact on neurovirulence. We speculate that the role of HE might be to assist in spread in the CNS and that a JHM ancestor once expressed HE at levels more similar to MHV-S. MHV-4 (from which rJHM was derived) was obtained from an encephalitic mouse (4, 13) and was subsequently serially passaged in the mouse brain (67-68). If higher HE expression was useful for neuropathogenesis one might expect passaging in the brain to select for high HE expression; however, expression could have since been downregulated upon tissue culture passaging. If expression of HE does not confer a fitness advantage it is surprising that expression has been maintained, even at low levels. For rA59, an MHV strain for which HE does not provide an advantage, there is a strong selection against HE expression when passaged in tissue culture (43). In the case of JHM, there are reports that passaging of JHM in DBT cells actually leads to selection for enhanced HE expression (47). It would be important to compare evolution of HE expression in a controlled experiment using both strains in parallel. It is possible that HE
expression is not relevant to virulence in the brain, but is more important in another organ system, such as the gastrointestinal track, where Neu4,5Ac₂ residues are more prevalent (56). Another possibility is that the virulence assay was not sensitive enough to detect a small differences between icJHM and icJHM/HE⁺ in the already virulent icJHM background; however, assays done by intranasal inoculations are usually capable of detecting slight differences in virulence.

Another possibility is that HE may enhance neurovirulence in a way that is redundant or unnecessary in the context of JHM, making HE expendable to JHM, but valuable to rA59-JHMS-HE⁺. For example HE could expand the tropism of rMHV-JHMS-HE⁺ by allowing it to bind cells that it otherwise would not, while icJHM may already be able to infect these cells by some other mechanism. Another possibility is that in the rMHV-JHMS-HE⁺ background HE might allow the virus to enter cells that a virus of this background can replicate in, while icJHM may not be able to replicate in such cells, even if HE promotes or enhances entry.

Detection of mRNA2-1 expression by rA59 was surprising because it has previously been reported not to by ³²P metabolic labeling experiments and PCR (41, 59, 72) and RT-PCR (Leibowitz in press). Not surprising that metabolic labeling does not detect a low abundance mRNA. We were able to confirm the rA59 qRT-PCR product was mRNA2-1 through sequencing, so the difference between our results and those prior is due to assay sensitivity. Though transcripts are expressed, a premature stop codon suggests that the only peptide that could be translated is 14 amino acids in length. It is surprising that rA59 expresses mRNA2-1, while icJHM/HE⁺ does not, since they both contains the same A to G TRS mutation that was reported to prevent transcription (59, 72) as well as the surrounding intergenic sequence (fig. 4-2). It is likely that this mutation reduces but does not abolish mRNA2-1 transcription and that the TRS mutation in combination with a sequence difference outside the intergenic region is responsible for the lack of mRNA2-1 transcription in icJHM/HE⁺ infected cells. This may be due to differences in the number of UCUAA repeats in the leader sequence; it was previously demonstrated that wild type JHM strains contain three UCUAA repeats, and reported that mutants that contain two repeats
express more mRNA2-1 and protein (59, 72-73). The wild type JHM viruses used here (icJHM, rJHM, and MHV-4) all have three UCUAA repeats, while rA59, and those viruses of the rA59 background (rMHV-JHMS-HE and rMHV-A59S-HE) have two UCUAA repeats (47, 59, 72-73). It could be that the combination of having three UCUAA repeats and a TRS mutation may completely block HE transcription for icJHM/HE+, but the presence of two UCUAA repeats for rA59 may allow some transcription in spite of the TRS mutation. The strong expression by rMHV-JHMS-HE+ and rMHV-A59S-HE+ can be explained because they have a “repaired,” JHM homologous, TRS sequences (34, 43) as well as two UCUAA repeats.

An interesting observation made in the course of these studies is that all published amino acid sequences for MHV HEs (except the infectious clone version of A59 and MHV-RI) contain the peptide AMAPRTLLL near the N terminus. It is intriguing because this peptide is 100% homologous to the mouse Qdm peptide. The Qdm peptide is derived from the signal sequence of certain MHC class Ia proteins (2, 14, 20). After the signal sequence is removed from MHC class Ia molecules the Qdm sequence is loaded onto Qa-1, a MHC class Ib (nonclassical MHC) molecule (2, 14, 20). Qdm bound Qa-1 serves as an inhibitory ligand for natural killer (NK) cells, while the absence of Qdm greatly enhances the susceptibility to NK mediated lysis (7, 9, 42, 66). The absence of Qdm indicates that the cell is not expressing MHC class Ia. The down regulation of MHC expression is a mechanism used by some viruses (1) and cancers to evade detection by T cells (54). While there is no evidence that MHV down regulates MHC class Ia to avoid immune detection, it is of interest that all MHV strains retain this sequence and even viruses, such as rA59, which cannot express full length HE, may still be capable of producing this peptide.

It is important to note that it was discovered after the construction of the icJHM viruses that the rJHM sequence on which it was based has a chimeric HE where last 193nt correspond to the A59 HE sequence. This results in three amino acid differences. Another rJHM isolate recovered by targeted recombination that has the full JHM HE displayed similar virulence and esterase activity.
to that expressing the chimeric HE protein (data not shown), suggesting that the chimeric nature of the icJHM HE did not affect the results of these experiments.

A reverse genetics system in which the entire JHM genome is cloned into bacterial plasmids has proven to be a useful and easily manipulated system for studying pathogenic determinants of MHV. By comparing wild type icJHM with that of icJHM/HE⁻ we have demonstrated, perhaps surprisingly that HE protein is not an important virulence determinant in the CNS.

Figure 4-5: Virulence of icJHM and icJHM/HE⁻. (A) Mice were intracranially inoculated with 10 PFUs of icJHM, icJHM/HE⁻, and rJHM (5 mice per group). (B) Mice were inoculated intranasally with 2000 PFUs of icJHM, icJHM/HE⁻, and rJHM (5 mice per group). There were no statistically significant differences after intracranial or intranasal inoculation (Gehan-Breslow-Wilcoxon test).
REFERENCES


Chapter Five

SEQUENCE ANALYSIS OF JHM AND A59 GENOMES AND ALIGNMENT OF NUCLEOCAPSID AND HEMAGGLUTININ-ESTERASE PROTEINS
INTRODUCTION

Sequencing of full genomes of various strains can assist in the identification of genetic causes of pathogenic differences. Recently The Institute for Genomic Research (TIGR) sequenced several relevant MHV genomes. These include rA59 (9) and rJHM.SD (7).

The comparison of individual gene sequences can be of benefit as well. If pathogenic differences have been mapped to a particular gene, comparing the sequence of this gene to other MHV strains may help identify amino acids responsible for the differences. Additionally the identification of consensus sequences and hypervariable domains can give insight into the importance of various residues and even the structure and function of the protein.

COMPARISON OF A59 AND JHM GENOMES

We have been studying the genetic determinants of the virulence differences between rA59 and rJHM.SD, and have compared their sequences to identify important genetic differences might be. Differences were spread throughout the genome and there were coding differences between genomes for all genes. The biggest differences were the deletion in the rA59 S gene relative to rJHM.SD, the disruption of the rA59 ORF4 gene, the mutations abolishing HE expression in rA59, and the mutation abolishing I protein expression in rJHM.SD. A59/JHM differences are summarized in Table 5-6.
Table 5-1: rJHM.SD compared to rA59. *Note that the ORF4 gene of rA59 is expressed as two separate reading frames due to a frameshift mutation. The 52 amino acid differences between ORF4 of rJHM and rA59 reflect a comparison of ORF4 of rJHM and ORF4a of rA59 and include differences due to rJHM ORF4 encoding a longer protein.

PROTEIN ALIGNMENTS

We aligned the N sequences of various strains and found the protein to be highly conserved (fig. 5-1). A similar alignment, with fewer strains, was done previously by M. Parker and P. Masters (8). Expression of JHM N confers a more virulent phenotype in the CNS than expression of A59 N; however this sequence alignment provided no further clues as to what residues may be important for virulence differences. This comparison may become useful later if important residues to N’s functions are identified, since strain differences in these residues may explain strain differences in phenotype. If various N proteins are compared for pathogenic differences sequence alignments may show residues or regions that correspond to certain phenotypes.

We also aligned the HE sequences of various strains (fig. 5-2). While some of these strains express full length HE, several of them have truncated ORFs due to premature stop codons.
Those that express full-length HE include rJHM.SD, rJHM.IA, MHV-S, DVIM, and MHV-MI. Those that do not express full-length HE include rA59-R13, rA59-Siddell, MHV-1, MHV-2, MHV-3, and MHV-RI. When there was more than one differing sequence available for the same virus we included both of them in our comparisons. This was the case for MHV-S, DVIM, and MHV-2.

One interesting observation we noted was that the DVIM HE sequence from Genbank number AB008939 (DVIM AB fig. 5-2) was identical to the MHV-S HE sequence from Genbank number AY771997 (MHV-S A fig. 5-2). Another sequence for MHV-S HE (Genbank number M64316, MHV-S M fig. 5-2) is consistent with MHV-S AY771997. DVIM has been sequenced two other times, both of which were 100% identical to each other and highly divergent from MHV-S (Genbank numbers AY771998 and AF091734, DVIM YF fig. 5-2). This suggests that DVIM AB008939 is misidentified and is really a MHV-S HE sequence.

Also of interest is that the HE proteins of most MHV strains contain the sequence AMAPRTLLL at the N-terminus (fig. 5-2). This sequence is 100% identical to the Qdm, which is a peptide derived from the signal sequence of certain MHC class Ia proteins (1, 4-5). This peptide is cleaved from MHC class Ia and loaded onto Qa-1, a MHC class Ib (nonclassical MHC) molecule (1) (4-5). Whether Qa-1 is bound to Qdm serves as a signal as to whether MHCs are being presented. Qdm bound Qa-1 serves as an inhibitory ligand for natural killer (NK) cells, while the absence of Qdm greatly enhances susceptibility to lysis (2-3, 6, 10). The homology of this conserved region of HE with Qdm could indicate a role in evading NK mediated lysis. Even those strains of MHV that do not express full-length HE could theoretically still express small peptides containing AMAPRTLLL because it is found after potential start sites and before premature stop codons in these viruses (fig 5-2). We only found two viral sequences that did not encode for this peptide; these were MHV-RI (fig. 5-2) and the A59 infectious clone virus (icA59; (11) ;not shown in alignment). MHV-RI encodes CIAPRTLLL instead while icA59 encodes AMAPLTLLL.
Figure 5-1: Nucleocapsid Protein Alignment.
Figure 5-1: Nucleocapsid Protein Alignment. Note on virus names: rA59-Siddell is abbreviated as A59-Sidd. MHV-2 is the TIGR sequence, MHV-2 A0 is MHV-2 Genbank number AF061835. DVIM AY7 is DVIM Genbank number AY771998, DVIM L37 is Genbank number L37760. Also note that the 3' end of DVIM AY7 is not deleted, but instead not sequenced. Similarity: Regions highlighted in light gray are similar, those highlighted in dark gray are somewhat similar, and those highlighted in black are non-similar.
Figure 5-2: Hemaglutinin-esterase protein alignment. The conserved Qdm sequence (AMAPRTLLL) is underlined. Note on names: rA59-Siddell is abbreviated A59-Sidd, MHV-S A is Genbank number AY771997, MHV-S M is Genbank number M64316, MHV-2 is the TIGR sequence, MHV-2 L is genbank number M64314, DVIM AB is Genbank number AB008939 and most likely is an incorrectly identified MHV-S sequence, and DVIM AF corresponds to Genbank numbers AY771998 and AF091734. Similarity: Regions highlighted in light gray are similar, those highlighted in dark gray are somewhat similar, and those highlighted in black are non-similar.
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Chapter Six

DISCUSSION
GENERAL CONCLUSIONS

Using the available reverse genetics systems and by generating a new infectious clone system for JHM.SD we have investigated the roles of several viral genes in conferring the pathogenic differences between mouse hepatitis virus (MHV) strains A59 and JHM.SD. A59 is weakly encephalitic and moderately hepatovirulent (10, 27). JHM.SD is highly encephalitic and nonhepatotropic (2, 24, 27). Previous studies demonstrated that spike (S) is a major determinant of strain differences in virulence, but also that there are other virulence determinants located within the 3' end of the MHV genome (6, 24, 27-28, 30). We focused our efforts on defining the roles of nucleocapsid protein (N), internal (I) protein, and hemagglutinin-esterase protein (HE) in pathogenesis.

We determined that N is a major contributor to strain specific differences in neuropathogenesis. JHM.SD N expression within the rA59 background conferred a highly neurovirulent phenotype to an otherwise weakly neurovirulent virus, while A59 N expression within the rJHM.SD background was neuroattenuating. We also determined that N is not responsible for the differences in hepatotropism. A59 N did not confer hepatotropism when expressed within the rJHM.SD background and conversely, JHM.SD N did not restrict hepatotropism when expressed from within the rA59 background. Nevertheless, JHM.SD N expression from within the rA59 background was slightly attenuating for replication in the liver.

While we did not discover a role for the HE and I proteins in pathogenesis, it cannot be concluded that these proteins are of no importance. It is possible that the affects of these proteins are too subtle to be measurable with our assays. It is also possible that the importance of these proteins would manifest themselves under other conditions. For example, these proteins could be more important under different routes of inoculation, in different organs, in different mouse strains, and/or in mice of different ages. Furthermore,
HE and I may not be relevant to rJHM.SD and rA59 pathogenesis, however they may provide important functions for other MHV strains that express them.

NEUROVIRULENCE

Viral Spread
The extent to which virus spreads in the CNS correlates with neurovirulence differences between rA59 and rJHM.SD. Our lab has shown that rJHM.SD spreads more extensively than rA59 in the CNS (6, 18, 22, 24) and neuronal cultures (2). S was shown to be a major contributor to spread differences between A59 and rJHM.SD (2, 6, 24, 27); JHM.SD S confers enhanced spread while A59 S expression reduces spread in the CNS. JHM.SD S also enhances spread in primary hippocampal neuron cultures. However, rA59/S\textsubscript{JHM} does not spread as well as rJHM.SD in the brain or in neuron cultures, suggesting other proteins are involved in spread differences (2, 6).

There is some evidence that N plays a role in neuronal spread. JHM.SD N was found to associate with microtubules as viewed in electron micrographs, which could suggest a role in rapid axonal transport (26). In addition N shares homology with the microtubule binding protein tau (26). Brains from animals infected with virus expressing JHM.SD N stain more robustly for antigen than those infected with viruses expressing A59 N. However, differences in N had no affect on spread in neuron cultures. This suggests, not surprisingly, that the mechanism by which N enhances antigen distribution in the brain is different from that of S. This also suggests that if N is involved in trafficking or fast axonal transport that this functionality of N is likely not different between rA59 and rJHM.SD.
Another difference between A59 and JHM in terms of spread is that some variants of JHM, including JHM.SD, are able to spread from cell-to-cell in the absence of CEACAM1a, while A59 cannot. Interestingly, measles virus in subacute sclerosing panencephalitis (SSPE) patients is also able to spread without receptor, and it was proposed that this may be accomplished through synaptic vesicles (11). It is unclear if JHM.SD is using a similar mechanism as that proposed for measles, or if it is using an alternative receptor. While there is indirect evidence that neurons express low levels of the CEACAM1a receptor, this has never been conclusively demonstrated (Bender et al., under review for JV). In the presence of very low CEACAM1a expression, the ability to carry out CEACAM1a independent spread would be advantageous. Thus, the ability of rJHM.SD to spread in the absence of CEACAM1a could contribute to the high neurovirulence of rJHM.SD. However, the ability of rJHM.IA to spread robustly in the CNS despite the fact it is incapable of CEACAM1a independent spread suggests the ability to spread without CEACAM1a is not crucial for high neurovirulence. Furthermore, the ability of JHM to spread efficiently among neurons may not be the same phenomenon as receptor independent spread observed in cell culture. The importance of the contribution of CEACAM1a independent spread to neurovirulence is difficult to determine because the JHM is so neurovirulent that the effect of attenuating mutations may not have observable phenotypes. The ability to spread in the absence of CEACAM1a−/− was attributed to JHM.SD S, as demonstrated by the ability of rA59/SJHM to cause lethal disease in CEACAM1a−/− mice (22) and spread in CEACAM1a−/− neurons (Bender et al., under review for JV).

A major difference between A59 and JHM.SD is that JHM.SD expresses HE while A59 does not, and this could play a role in spread differences. HE could act as a second attachment protein, perhaps enhancing spread or broadening cell types infected. It was demonstrated that MHV-S HE when expressed in the rA59 background enhanced spread
in the CNS, but only if JHM.SD S rather than A59 S was also expressed (7, 14). This was shown not to rely on the esterase activity, so is presumed to rely on the binding activity. This would support the idea that HE acts as a second attachment protein. It is possible that through its binding activity, HE could assist JHM by attaching to the cells, which would be particularly advantageous when utilizing low abundance (i.e. CEACAM1a of neurons) or inefficient receptors (i.e. alternative receptors). The fact that HE only conferred enhanced spread when expressed in combination with JHM S, may suggests that JHM S, and not A59 S, can mediate infection in cells for which HE enhances binding.

Given that rMHV-JHMS-HE+ and rMHV-JHMS-HE− spread more extensively than rMHV-JHMS-HE (7) it was surprising that mutations that ablated HE expression in icJHM had no effect on virulence, even following IN inoculation, a condition under which subtle differences can often be measured. Since no difference was measured it would imply that JHM.SD HE does not contribute significantly to neurovirulence. Since rJHM.SD (and parental wt MHV-4) express lower levels of HE, it might be that HE expression by JHM.SD is not high enough to have an impact on neurovirulence.

There is much contradictory evidence in the literature about the importance of HE to MHV infection. When HE is expressed within the A59 background it is quickly selected against in tissue culture(14), other reports concluded that passaging of JHM in rat neuron cultures or DBT cells selected for HE expression (19, 37). There are contradictory reports regarding the relationship between HE expression and neurovirulence (9, 39-40, 43). Since these studies were carried out before reverse genetics systems were available, the viruses analyzed were not isogenic and may have had other genetic differences that could explain differences in pathogenesis. Comparisons of rMHV-SJHM-HE+ and rMHV-SJHM-HE− suggest that expression of HE can be important to neurovirulence (7), but comparisons of icJHM and icJHM/HE− suggest that HE expression is not be important for JHM.SD neurovirulence.
**Viral Replication**

Interestingly there is no direct correlation between viral titer in the CNS and disease. rA59 and rJHM.SD replicate to similar titers in the CNS, but there is very little antigen detected in A59 infected brains compared to robust JHM antigen expression (2, 6, 18, 22, 24). rA59/S_{JHM} spreads more robustly than rA59 in the CNS, but replicates to lower titers (27). JHM also replicates to lower titers than A59 in tissue culture. It is likely that JHM produces less infectious virus per cell than A59, but its ability to spread to and infect more cells makes up the difference in CNS titers. The lower titers from JHM are thought to be due to an unstable S, which can lose the S1 subunit, which inactivates the virions; in support of this hypothesis JHM has a higher particle to PFU ratio (38). Also in support of this idea, when expressed within the A59 background JHM S confers replication to lower titer in tissue culture (2, 27).

Interestingly rA59/N_{JHM} replicated to higher titers in the CNS than either rJHM or rA59. This is likely due to a combination of expression of JHM N which leads to the infection of more cells in the CNS, and A59 S which confers efficient replication. On the other hand rJHM/N_{A59} both spreads less well than rJHM in the CNS and replicates to lower titer due to the less stable spike.

**T cell response**

JHM induces a much weaker T cell response than A59 in the CNS, though both viruses induce a T cell response after intraperitoneal inoculation (17), suggesting these differences are unique to the response in the brain. The T cell response is necessary for clearance of the virus from the CNS. A weaker T cell response could contribute to the more robust antigen spread in the brain and the resulting enhanced lethality. The inability to induce a robust T cell response does not map to the JHM S, but rather to the JHM background genes. Expression of JHM N appears to slightly reduce the T cell response.
to rA59. This was barely significant and it is unclear how such a slight reduction in T cell response could affect virulence.

Differences in T cell response between JHM and A59 appear to be due to CD8 T cell priming. Interestingly, rA59 is readily detectable in the CLN, the site of T cell priming for CNS infections, while JHM is not (17). rA59/N_{JHM} and rA59 had similar titers in the CLN (data not shown). This could suggest priming is not responsible for rA59 and rA59/N_{JHM} differences in T cell response, but cannot be ruled out because the simple presence of virus does not guarantee robust priming. However, the barely detectable titers of rJHM in the CLN could indicate weak priming. It would be interesting to examine the rJHM/N_{A59} titers in the CLN because the rJHM/N_{A59} and rJHM T cell response difference was more significant and the fact that rJHM/N_{A59} is able to induce a stronger T cell response could imply that more virus would be present in the CLN.

**Innate Immune Response**

rA59 and rJHM also induce different innate immune responses. rA59 infection induces more T cell chemoattractants and more IFN-γ (29, 34). The greater induction of T cell chemoattractants correlates with a greater T cell response and the greater IFN-γ induction is probably due to the presence of more T cells. Another difference is that of macrophage chemoattractants. JHM produces more and this correlates with more macrophages in the CNS, which have been suggested to be immunopathogenic (29, 34). rA59/S_{JHM} induced more macrophage chemoattractants and recruited more macrophages into the CNS than rA59, but not as much as JHM, suggesting that S is partially responsible for differences in macrophage response (30).

Differences in IFN response could also lead to differences in lethality and spread of virus, though the data does not support this idea. In data obtained in our lab JHM and A59 appear to induce similar IFN-β responses (34); however, Rempel et al, reported that JHM
induces a stronger IFN-β (29). In addition there is no difference between A59 and JHM in interferon sensitivity during infection of L2 cells (32) and preliminary evidence suggests that there is no difference in interferon sensitivity between A59, JHM, and rA59/N_JHM in bone marrow derived macrophages (unpublished).

**Role of internal protein in pathogenesis**

The I protein is encoded within the N gene. Thus, differences in neurovirulence due to N gene exchanges could have been due to differences in I protein. However, Alb110, an A59 mutant that does not express I protein, exhibited no difference in neurovirulence compared to wild type control, Alb111. This suggests that JHM N, and not the lack of I expression, was responsible for the enhanced neurovirulence of rA59/NJHM over rA59.

**HEPATOVIRULENCE**

**Importance of spike in pathogenesis**

A59 infects the liver and causes hepatitis, but JHM is not able to replicate in the liver (2, 24). The inability of JHM to replicate in the liver is unlikely due to a block in entry. Expression of A59 S did not confer replication in the liver to rJHM/S_A59; nor does expression of JHM S prevent replication in the liver to rA59/S_JHM (24).

**Importance of Fibrinogen-like protein 2 to Liver Pathogenesis**

Fibrinogen-like protein 2 (fgl2) is produced in both a membrane bound and soluble form. The membrane bound form is a prothrombinase (cleaves prothrombin to thrombin) and causes fibrin deposition (5, 25), while the soluble form suppresses T cell proliferation and DC maturation (15-16). The ability to induce fgl2 has been demonstrated to be important in the development of hepatitis in several viral systems. The induction of fgl2 by
hepatitis-B virus and hepatitis-C virus in humans has been linked to fibrin deposition in
the liver and disease severity (4, 12, 20, 44). The MHV strains A59 and MHV-3 were
reported to induce fgl2 in the liver during infections of susceptible mice (5, 25). Blocking
fgl2 not only prevents hepatitis (1, 5, 13), but also, can lead to viral clearance from the
liver of MHV-3 infected susceptible mice (13). N of MHV-3 and A59 have been
demonstrated to induce fgl2, while N of JHM does not (3, 21, 25). Because of these data
we tested if JHM N was responsible for the block in JHM replication in the liver.

rA59/N_{JHM} replicates to lower titers and appeared to be slightly less virulent than rA59 in
the liver, but the expression of JHM N did not block replication (2). When fgl2 is blocked,
MHV-3 is still able to replicate in the liver (1, 5, 13), so perhaps it is not surprising that
expression of JHM N did not block replication. However, given the MHV-3 data, the fact
that rA59/N_{JHM} causes nearly the same degree of hepatitis as rA59 was unexpected. This
implies that A59 doesn’t need to induce fgl2 to cause hepatitis or that it induces fgl2
independent of which N it expresses. When we compared the ability of rA59, rA59/N_{JHM},
and rJHM to induce fgl2 in C57BL/6 macrophages, all viruses induced fgl2, but barely
over background. The weak induction of fgl2 may have been because we used bone
marrow derived macrophages while Q. Ning et al. did MHV-3 experiments using
peritoneal macrophages. The importance of fgl2 induction to rA59 induced hepatitis in
C57BL/6 mice is worthy of future investigation.

Roles of N and I to hepatitis

Though rA59/N_{JHM} replicates in the liver, it is slightly less virulent and its replication is
slightly reduced compared to rA59. rA59/N_{JHM}, in addition to expressing JHM N, does not
express I protein. We investigated whether I protein expression impacts hepatovirulence.
An A59 virus mutant unable to express I protein (Alb110) was just as virulent in the liver
as a wild type control (Alb111) and our rA59. rA59/N_{JHM} was less virulent than all three of
these viruses, however, this difference was not quite significant. This implies that the
difference between rA59 and rA59/N_{JHM} in the liver is more likely due to N, than I.

**Importance of Type I Interferon Response**

It is possible that the type I interferon response may contribute to the ability of A59 and
JHM to replicate in the liver. JHM is able to infect hepatocytes in tissue culture, and in
IFNAR\(^{-}\) mice JHM replicates in the liver (31). These data suggest that JHM is able to
enter and replicate in hepatocytes, and that the early host response is able to block JHM,
but not A59 infection of the liver. Other evidence for the importance of type I interferon
response to liver pathogenesis is that mutations in the predicted catalytic histidines of the
putative 1",2"-cyclophosphodiesterase (CPD) domain (36) of A59 ns2 increase
sensitivity of virus to type I IFN in bone marrow derived macrophages (unpublished).
These same mutations confers very poor replication in the liver, but not the brain (33).
Though ns2 is important to replication in the liver, it is unlikely that differences in ns2 are
responsible for differences in JHM and A59 liver tropism because repJHM-RA59 can
replicate in the liver even though it expresses JHM ns2, and repA59-RJHM cannot
replicate in the liver even though it expresses A59 ns2 (23).

**FUTURE DIRECTIONS AND FINAL COMMENTS**

**Mapping of virulence determinants**

The JHM infectious clone developed here along with the previously developed A59
infectious clone allows for reverse genetics of the entire genomes of JHM and A59. This
can be used to help map other determinants of strain differences in pathogenesis. When
the first 2/3 of the genome was exchanged between A59 and JHM pathogenic differences
were largely mapped to the 3’ 1/3 of the genome (23). However, we have since observed that the virus expressing the A59 replicase proteins and ORF2a and the JHM 3’sequence (repA59-RJHM) kills mice 1 to 2 days faster than rJHM when infected intracranially with 10 PFU (unpublished), while the converse chimeric virus (repJHM-RA59) was attenuated compared to rA59 after intranasal inoculation (unpublished). Additionally, in a pilot experiment repA59-RJHM induced a stronger T cell response than rJHM. These observations need to be confirmed with further experiments. If there are differences in virulence due to replicase and/or ORF2a, A59/JHM chimeras can be generated using the JHM and A59 infectious clones to map the genes responsible.

In addition to there being potential differences in virulence between JHM and A59 located within the replicase gene, the functions of several of the proteins encoded by the replicase gene are not known. Enzymatic activities have been attributed to some of these proteins, but even in cases where an enzymatic activity has been identified, such as with ADRP of nsp3, its function in the viral lifecycle may not known be known. icJHM and icA59 can be used to mutate residues predicted to be important in these genes to help explore their function. Additionally, icJHM can be used to determine what role, if any, the replicase and ORF2a genes play in virulence differences between JHM variants and icA59 can similarly be used to map genes responsible for differences in A59 variants.

It is likely that other proteins responsible for important virulence differences between A59 and JHM are located in the 3’ third of the genome. rA59/SJHM/NJHM, was almost as virulent as rJHM, but still killed mice significantly slower. Also, unlike rJHM, rA59/SJHM/NJHM produced a robust T cell response (though it was reduced compared to rA59/SJHM), suggesting another protein(s) in the JHM background likely contributes to enhanced neurovirulence and/or a diminished T cell response. Also we have still not identified the gene(s) responsible for differences in liver tropism between A59 and JHM.
Data shown here suggests that differences in N, I, and HE genes are not responsible for differences in hepatotropism. Previous data ruled out the replicase, ORF2a (23), and S genes (24), thus we have narrowed the candidate genes capable of conferring differences in hepatotropism down to M, E, and ORF5a or a combination thereof. Interestingly, ORF5a has recently been shown to be an interferon antagonist (8).

Differences in ability to resist host immune response could be responsible for differences in organ tropism, making ORF5a a promising candidate for the gene responsible for A59/JHM differences in liver tropism. The basal expression of interferon stimulated genes (ISGs) are higher in the liver than the brain (unpublished), which could suggest that the ability to resist IFN is more important in the liver. It would be of interest to exchange the M, E, and ORF5a genes between rA59 and rJHM to explore their contributions to neurovirulence and hepatotropism.

**Determining the mechanism by which nucleocapsid protein contributes to virulence differences.**

While we showed that enhanced virulence due to JHM N correlates with greater replication and spread in the CNS, the exact mechanism by which JHM N causes these differences was not found. One possibility worth further exploration is that JHM N may alter the innate immune response. Differences in the cytokine profiles and the innate immune infiltrate of JHM and A59 infected brains have previously been identified (6, 29-30, 34). Infections with chimeric recombinant viruses differing in N expression can be used to test whether N differences contribute to A59/JHM differences in innate immune response. If the expression of different N proteins alters the immune response, it could impact virulence by changing the ability of the host to control infection and/or by leading to immunopathogenesis.

It would also be of interest to map the amino acid residues responsible for differences between rA59 and rJHM neurovirulence. The location and type of residue may help
predict the functional differences responsible for virulence differences. Mapping of the important residues could be done by exchanging regions of the A59 N gene for those of rJHM. Examining the affects of other MHV N genes on neurovirulence can help determine which residues are likely to be important for virulence differences. For example the N from MHV-1, a pneumotropic strain that replicates poorly in the CNS, when expressed in the rA59 background did not significantly alter neurovirulence (unpublished). This would suggest that residues of JHM that are different from A59 but shared with MHV-1 are less likely to be responsible for enhanced neurovirulence than those residues only found in JHM.

Further exploration of the importance of I protein to virulence

Expression of the I protein did not appear to be important to virulence since there were no detectable differences following intracranial and intranasal infections with Alb110 and Alb111. A potential complication is that these viruses were more neurovirulent than our rA59 isolate. Slight differences in virulence are typically more easily detected in less virulent backgrounds. It would be preferable to compare isogenic viruses differing in expression of the I protein in the context of our rA59 background. The I mutation in our rA59 background could then be used to compare the ability of the I mutant to spread to the liver after intracranial inoculation. We could also compare neurovirulence after intranasal inoculation, which can be more sensitive to strain differences in virulence. A more sensitive assay might be an in vivo competition experiment. We could mix Alb110 and Alb111 at a 1:1 ratio and inoculate into the brain or liver. We could then isolate virus from these organs and determine if one outgrew the other; this could possibly be done using Taqman qRT-PCR with a probe that distinguishes between the sequences.

Further exploration of the importance of HE protein to virulence

The HE protein does not appear to be an important contributor to the high neurovirulence of icJHM. It would be interesting to determine if HE has an impact on JHM neurovirulence
when expressed at higher levels. There are JHM variants that differ in HE expression, but they are not isogenic with our icJHM. The number of UCUAA repeats in the JHM leader sequence correlates with HE expression levels (35, 41-42). Wild type JHM strains contain three repeats and are low HE expressers, while strains that contain two repeats and are high expressers have been selected. If this is correct we should be able to use the JHM infectious clone to generate HE⁺ and HE⁻ viruses in which the leader has been mutated to contain two UCUAA repeats. This would allow comparison of a high HE expresser and a nonexpresser with isogenic backgrounds, in addition to confirming the role of UCUAA repeats in transcriptional regulation. In addition to these experiments an in vivo competition assay between icJHM and icJHM/HE⁻ could be used to determine if icJHM has a selective advantage over icJHM/HE⁻. We attempted to use an enzymatic assay to distinguish HE positive and negative plaques, but this proved unreliable. Taqman qRT-PCR could be used if an appropriate probe could be designed to distinguish the single nucleotide difference between the TRS of icJHM and icJHM/HE⁻, but likely PCR would need to be done on individual plaques to determine if HE mRNA is produced.

**Final Comments**

The availability of reverse genetics systems has proven invaluable to the study of genetic determinants of virulence. We have determined that strain specific differences in N can have a dramatic impact on neurovirulence. Though we were unable to determine the mechanism by which N impacts neurovirulence, we know it is associated with greater replication and wider antigen distribution in the brain. Strain specific differences in N also affect replication in the liver, but only appear to be of minor importance to liver pathogenesis. We also studied the impact of abrogation of expression of HE or I proteins on pathogenesis and were unable to observe a phenotype, though our data would suggest they are not of significant importance to strain differences in neuro- and hepatovirulence. Further research into genes responsible for strain differences in
virulence will help elucidate mechanisms by which viruses induce hepatitis and encephalitis and these studies in the long term can contribute to the understanding of the mechanisms of human disease and treatment strategies.

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