JOURNAL OF VIROLOGY, July 2005, p. 9108–9118 Vol. 79, No. 14
0022-538X/05/$08.00+0 doi:10.1128/JVI.79.14.9108–9118.2005

Contributions of the Viral Genetic Background and a Single Amino Acid Substitution in an Immunodominant CD8$^+$ T-Cell Epitope to Murine Coronavirus Neurovirulence

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Received 13 January 2005/Accepted 30 March 2005

The immunodominant CD8$^+$ T-cell epitope of a highly neurovirulent strain of mouse hepatitis virus (MHV), JHM, is thought to be essential for protection against virus persistence within the central nervous system. To test whether abrogation of this H-2D^b-restricted epitope, located within the spike glycoprotein at residues S510 to 518 (S510), resulted in delayed virus clearance and/or virus persistence we selected isogenic recombinants which express either the wild-type JHM spike protein (RJHM) or spike containing the N514S mutation (RJHM$_{N514S}$), which abrogates the response to S510. In contrast to observations in suckling mice in which viruses encoding inactivating mutations within the S510 epitope (epitope escape mutants) were associated with persistent virus and increased neurovirulence (Pewe et al., J Virol. 72:5912–5918, 1998), RJHM$_{N514S}$ was not more virulent than the parental, RJHM, in 4-week-old C57BL/6 (*H-2^b*) mice after intracranial injection. Recombinant viruses expressing the JHM spike, wild type or encoding the N514S substitution, were also selected in which background genes were derived from the neuroattenuated A59 strain of MHV. Whereas recombinants expressing the wild-type JHM spike (SJHM/RA59) were highly neurovirulent, A59 recombinants containing the N514S mutation (SJHM$_{N514S}$/RA59) were attenuated, replicated less efficiently, and exhibited reduced virus spread in the brain at 5 days postinfection (peak of infectious virus titers in the central nervous system) compared to parental virus encoding wild-type spike. Virulence assays in BALB/c mice (*H-2^d*), which do not recognize the S510 epitope, revealed that attenuation of the epitope escape mutants was not due to the loss of a pathogenic immune response directed against the S510 epitope. Thus, an intact immunodominant S510 epitope is not essential for virus clearance from the CNS, the S510 inactivating mutation results in decreased virulence in weanling mice but not in suckling mice, suggesting that specific host conditions are required for epitope escape mutants to display increased virulence, and the N514S mutation causes increased attenuation in the context of A59 background genes, demonstrating that genes other than that for the spike are also important in determining neurovirulence.

Coronaviruses are enveloped RNA viruses with large nonsegmented genomes that commonly infect the respiratory and gastrointestinal tracts of domestic animals and humans. Disease induced by coronaviruses can range in severity from mild to severe and potentially lethal. For example, human coronaviruses 229E and OC43 induce mild respiratory disease, while the emergence of a novel human coronavirus, severe acute respiratory syndrome (SARS) coronavirus, as the etiologic agent of SARS demonstrates that a coronavirus can also cause a serious, potentially lethal infection in humans (15). Likewise, while the highly neurovirulent strain of MHV, strain JHM, which is also referred to as MHV-4 in the literature (4), was isolated from a mouse with hind limb paralysis and causes acute, lethal encephalitis in C57BL/6 (B6) mice, the laboratory-adapted strain of MHV, strain A59, induces only mild encephalitis but results in chronic primary demyelination. Understanding the determinants of virulence and virus persistence are imperative to a full understanding of coronavirus pathogenesis.

Intracranial infection with A59 results in virus titers that peak on day 5 postinfection (p.i.), with virus clearance occurring by days 10 to 12 p.i; but, despite efficient clearance of infectious A59, chronic demyelination ensues, typically peaking in severity at 4 weeks p.i (28, 44). JHM infection, however, results in fatal encephalitis during the first week of infection, with only the occasional survivor (43).

Persistent infectious virus cannot be isolated from mice infected with either A59 or JHM undergoing primary demyelination of the spinal cord. Interestingly, however, in one model system, mice do become persistently infected with JHM and infectious virus can be isolated from paralyzed mice that exhibit severe demyelination. This model involves JHM infection of 10-day-old mice that receive passive immunity by nursing on JHM-immune dams; thus, mice are protected from acute encephalitis by passive antibody transfer, but develop severe paralysis at 3 weeks p.i. accompanied by persistent virus (41).

Virus persistence is dependent on several viral and host

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factors, but a requirement in establishing a persistent infection is the ability to avoid immune surveillance; the central nervous system (CNS) is thought to be a reservoir for persistent viral infections due to unique regulatory mechanisms intended to protect it from potential immune-mediated damage. A strategy of mutation is a means by which RNA viruses can passively escape the adaptive immune response. Due to the error-prone RNA-dependent RNA polymerase, coronaviruses, like other RNA viruses, often exist inside the cell as a quasispecies or a population of virus variants (12–14). In addition, coronaviruses have a well-documented ability to undergo homologous recombination (24, 27, 48, 49), which generates further variation on which selection pressures can act.

In the suckling model of JHM-induced demyelination which results in virus persistence, virus obtained from animals with severe paralysis reveal a variety of amino acid substitutions in the immunodominant $CD8^+$ H-$2D^b$-restricted S510 epitope, with the most common being a mutation in the anchor residue, N514S, which prevents binding to the major histocompatibility complex molecule (41). The S510 epitope (amino acids 510 to 518) is located within the hypervariable region (HVR) of the S1 subunit of the spike protein (3). Infection of suckling mice with virus isolates containing the epitope mutations results in an increase in morbidity and mortality compared to suckling mice infected with the parental strain of JHM (42). Thus, escape from the S510 epitope of JHM has been interpreted to be a major determinant of virulence, viral persistence, chronic infection of the spinal cord, and disease in the suckling mouse model. However, it still remains unclear whether other mutations are involved in establishing persistence. It has not been established whether the S510 epitope is required for virus clearance since the H-$2K^b$-restricted S598-605 (S598) epitope does not undergo mutation during infection. In addition, some strains of MHV, including the A59 strain and a variety of JHM mutants, have a deletion within the hypervariable domain of the spike protein and lack the S510 epitope yet are cleared efficiently and are, in fact, less pathogenic (10).

To explore the importance of the S510 epitope in virus clearance and pathogenesis, isogenic recombinant viruses containing either the wild-type JHM spike (RJHM) or the N514S substitution within the wild-type JHM genome ($RJHM_{N514S}$) were selected using targeted RNA recombination. In addition, recombinants containing the A59 background genes were selected which expressed either the JHM spike (SJHM/RA59) or the N514S-containing JHM spike ($SJHM_{N514S}$/RA59) in order to assess the phenotype of the N514S mutation in a more neuroattenuated background. The recombinant viruses containing the S510 mutation alone in the JHM background are not more virulent than the parental virus and the S510 mutation in the A59 background was slightly attenuated compared to recombinants with the wild-type JHM spike in 4-week-old mice after intracranial injection. Epitope mutants in the A59 background ($SJHM_{N514S}$/RA59) were not only slightly attenuated in B6 mice but were cleared from the brain with similar kinetics as the isogenic virus expressing the wild-type JHM spike (SJHM/RA59). Thus, escape from the S510-specific $CD8^+$ T-cell response does not necessarily provide a selective replication advantage or reduce viral clearance, and the N514S mutation plays a role in attenuation in adult/weanling 4-week-old mice. Attenuation due to the N514S mutation is not due to removal of an epitope that elicits an immunopathogenic immune response as the recombinant viruses had similar 50% lethal doses (LD_{50}s) in BALB/c mice, which do not recognize the S510 epitope. These results highlight the importance of the hypervariable domain of the spike protein in neurovirulence and add to a growing body of evidence that there are many factors that contribute to the persistence of virus in the CNS.

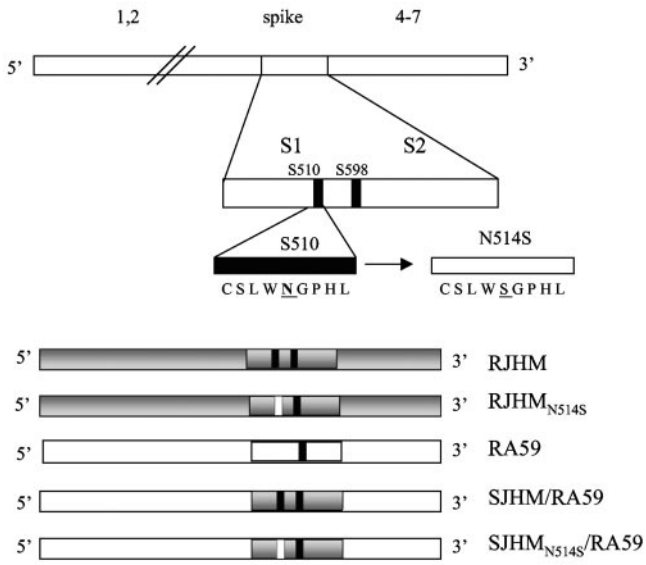

FIG. 1. Schematic diagram of recombinant viruses. Recombinant viruses were selected by targeted recombination as described in Materials and Methods. RJHM is wild-type recombinant and $RJHM_{N514S}$ is a recombinant containing the epitope mutation; SJHM/RA59 and $SJHM_{N514S}$/RA59 encode wild-type JHM spike and JHM spike with the N514S substitution, respectively, both with A59 background genes. RA59 contains all genes derived from A59 (29).

MATERIALS AND METHODS

Virus and cells. Wild-type JHM Iowa (JHM.IA) and v106, an animal isolate containing the N514S mutation that was derived from a mouse persistently infected with JHM.IA, were kindly provided by Stanley Perlman (University of Iowa). RJHM and $RJHM_{N514S}$ were selected by targeted recombination using as parental virus fMHV-JHM, clone B3b, and pJHM.SD for the transcription of synthetic mRNA (both obtained from Stanley Perlman, University of Iowa). These viruses contain the spike gene derived from JHM-MHV-4 (MHV-4 is also known as JHM.SD in the Perlman nomenclature) (38). Recombinants containing the JHM spike in the A59 background were selected using fMHV-A59 as a parental virus and pMH54-S4 (encoding the A59 genome with the JHM.SD spike) as previously described (43) (Fig. 1 for schematic). Two independent recombination events were carried out for the selection of all viruses. Recombinant viruses were isolated after two rounds of plaque purification on L2 cells. Virus stocks were then prepared on 17Cl-1 monolayers. The S gene of each recombinant was sequenced using previously described primers specific for the spike gene (43). All cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum on plastic tissue culture flasks.

PCR mutagenesis. PCR mutagenesis with the following primers replaced a single nucleotide, resulting in an asparagine to serine amino acid change: 5′-GTTCTCTTTGGAGTGGGCCCCATT-3′ and 5′-AATGGGGCCCACTCCAAAGAGAAC-3′ (8).

Inoculation of mice. C57BL/6 (B6) and BALB/c mice were purchased from the National Cancer Institute (Frederick, MD). Virus-free, 4- to 5-week-old, male mice were used in all experiments. Mice were anesthetized with isoflurane (IsoFlo; Abott Laboratories, North Chicago, IL) for all intracranial injections. Virus was diluted in phosphate-buffered saline containing 0.75% bovine serum albumin and a total volume of 25 μl was injected into the left cerebral hemisphere.

Virulence assays. Fifty percent lethal dose (LD_{50}) assays were carried out as described previously (23). Mice were inoculated with three or four dilutions of each appropriate virus. At least two independent experiments were performed for each virus in each strain of mouse and a total of five animals per dilution per virus were analyzed for each experiment. Mice were examined for signs of disease or death every day for 21 days postinfection. LD_{50} values were calculated by the Reed-Muench method (47).

Virus replication in mice. Mice were infected with 10 PFU of virus. On days 3, 5, 7, 12, and 28 days postinfection, mice were sacrificed and brains were obtained. Half of each brain was placed in isotonic saline with 0.167% gelatin (gel saline), weighed, and stored frozen at −80°C until virus titers were determined, while the other half was preserved for histology. Brains were homogenized and standard plaque assays were performed on L2 monolayers to determine titers as previously described (21). Occasionally, mice infected with low doses of virus (10 PFU or less) did not exhibit virus titers or virus antigen staining and did not develop encephalitis; it is assumed that these mice did not get infected and, thus, they were excluded from the titer averages.

Histology and immunohistochemistry. Mice were infected with 10 PFU of virus and brains were harvested from phosphate-buffered saline-perfused mice days 3, 5, and 7 p.i. The right hemisphere of the brain was placed directly in 10% phosphate-buffered formalin overnight. Tissue was then embedded in paraffin, sectioned, and left unstained for immunohistochemistry or stained with hematoxylin and eosin for histological analysis. Immunohistochemical analysis was performed to determine the level of viral antigen expression. A monoclonal antibody against the nucleocapsid protein of MHV, kindly provided by Julian Leibowitz (Texas A&M University), was used with an avidin-biotin-immunoperoxidase technique (Vector Laboratories). All slides were read in a blinded manner, at least four mice were examined from two separate experiments for each virus.

Splenocyte isolation. Six or seven days postinfection (depending on the virus used), mice were sacrificed and perfused with 10 ml of phosphate-buffered saline. Spleens were removed and placed in 5 ml 1% fetal bovine serum in RPMI. Spleens were homogenized in nylon mesh bags by gently rubbing the spleen with the plunger from a 10-ml syringe. Red blood cells were lysed by adding 1 to 2 ml ammonium chloride per spleen for approximately 1.5 minutes at which point 10 ml of 1% fetal bovine serum RPMI is added to stop the lysis. Lymphocytes are then washed two times in 1X phosphate-buffered saline and counted on a hemacytometer.

IFN-γ assay and flow cytometry analysis of T cells. Purified splenocytes isolated from infected mice were resuspended in 5% fetal bovine serum RPMI and assayed for gamma interferon (IFN-γ) secretion in response to peptide stimulation as described previously (35). Briefly, 10^6 splenocytes per well were cultured for 5 h at 37°C in 200 μl of 5% fetal bovine serum RPMI, 10 units human recombinant interleukin-2, and 1 μl/ml brefeldin A (Golgi stop, PharMingen). Cells were incubated with or without peptides which were used at a concentration of 0.1 μg/ml. Cells were then stained for surface expression of CD8a (clone 53-6.7) and CD4 (clone RM4-5), followed by fixation and permeabilization using the Cytofix/Cytoperm kit (PharMingen) and stained with fluorescein isothiocyanate-conjugated monoclonal rat anti-mouse IFN-γ antibody (clone XMG 1.2, PharMingen). Analysis was performed on a FACScan flow cytometer (Becton-Dickson).

RESULTS

Selection of recombinant viruses with inactivating amino acid substitutions in the immunodominant $CD8^+$ T-cell epitope S510. In order to assess the role of the S510 epitope in viral clearance and to understand the role of S510 epitope escape in establishing virus persistence and demyelination, we used targeted RNA recombination to select pairs of isogenic recombinant viruses differing only at spike residue N514, within the S510 epitope, in both JHM and A59 backgrounds (see Materials and Methods). Figure 1 depicts these recombinant viruses as well as the other viruses used in this study: RJHM, the recombinant wild-type JHM; $RJHM_{N514S}$, isogenic except for the N514S substitution; RA59, wild-type recombinant RA59 (also referred to in the literature as R13); SJHM/RA59, expressing the JHM spike in the A59 background (also referred to as R22); and $SJHM_{N514S}$/RA59, expressing the JHM spike with the N514S mutation in the A59 background. JHM.IA, a wild-type strain of JHM, and v106, isolated from a mouse persistently infected with JHM.IA and shown to contain the N514S mutation, were included in experiments as controls. Although JHM.SD is more virulent than JHM.IA in passively immunized pups in the suckling mouse model and this can be mapped to a single amino acid change at position S310 (38), the LD_{50} of JHM.IA and JHM.SD is similar in 4-week-old B6 mice used for these studies (as shown below).

N514S substitution abrogates the S510 epitope-specific $CD8^+$ T-cell response. To verify that $RJHM_{N514S}$ and $SJHM_{N514S}$/RA59 did not elicit a T-cell response to S510 in B6 mice an IFN-γ secretion assay was used to assess functional activation in response to viral peptide stimulation. $CD8^+$ T cells harvested from animals infected with the wild-type viruses, RJHM and JHM.IA, responded to both the S510 and S598 viral peptides (Fig. 2A). As expected, splenocytes isolated from mice infected with viruses encoding the N514S mutation did not produce IFN-γ in response to stimulation with the S510 peptide; however, approximately 2.1% and 4.8% of the $CD8^+$ T-cell population responded to the S598 peptide in animals infected with $RJHM_{N514S}$ and v106, respectively (Fig. 2A). Since these viruses were all highly lethal, causing extensive mortality on day 7, this assay had to be performed on day 6 p.i., 1 day before the usual peak of virus-specific $CD8^+$ T cells in the spleen.

Mice infected with the recombinants containing the A59 background genes uniformly survived the first week of infection, allowing analysis of the immune response on day 7 p.i. RA59 has an in-frame 52-amino-acid deletion within the hypervariable domain of the spike that encompasses the S510 epitope, thus animals infected with RA59 do not secrete IFN-γ in response to stimulation with the S510 peptide. Absence of an IFN-γ response to S510 confirmed that the N514S mutation eliminated S510 as a functional epitope in $SJHM_{N514S}$/RA59 (Fig. 2B). However, a response to both S510 and S598 was detected in SJHM/RA59-infected animals (Fig. 2B).

Inactivating mutations in the S510 epitope cause attenuation in mice. Standard 50% lethal dose experiments were performed to address the importance of epitope inactivating mutations in MHV pathogenesis. The virulence of the epitope escape mutants was first assessed in 4-week-old B6 mice and at least two LD_{50} assays were performed for all viruses examined and the data were pooled. Although still highly virulent, $RJHM_{N514S}$, the recombinant containing the targeted mutation, was slightly attenuated compared to wild-type RJHM (Table 1). Most striking is the observation that the animal isolate, v106, which was more virulent than JHM.IA when used to infect suckling mice (42) was attenuated compared to JHM.IA in 4-week-old mice. Thus, the epitope mutants were not more virulent than the wild-type parental viruses in weanling mice.

We have previously shown that the JHM spike is a major determinant of neurovirulence (43) and introduction of the JHM spike within the A59 genome results in a dramatic increase in virulence (Table 1). Although the LD_{50} of SJHM/RA59 is nearly identical to that of RJHM, animals infected with SJHM/RA59 consistently begin to die 3 to 4 days after animals infected with RJHM. The JHM spike is able to mediate membrane fusion with target cells in the absence of its

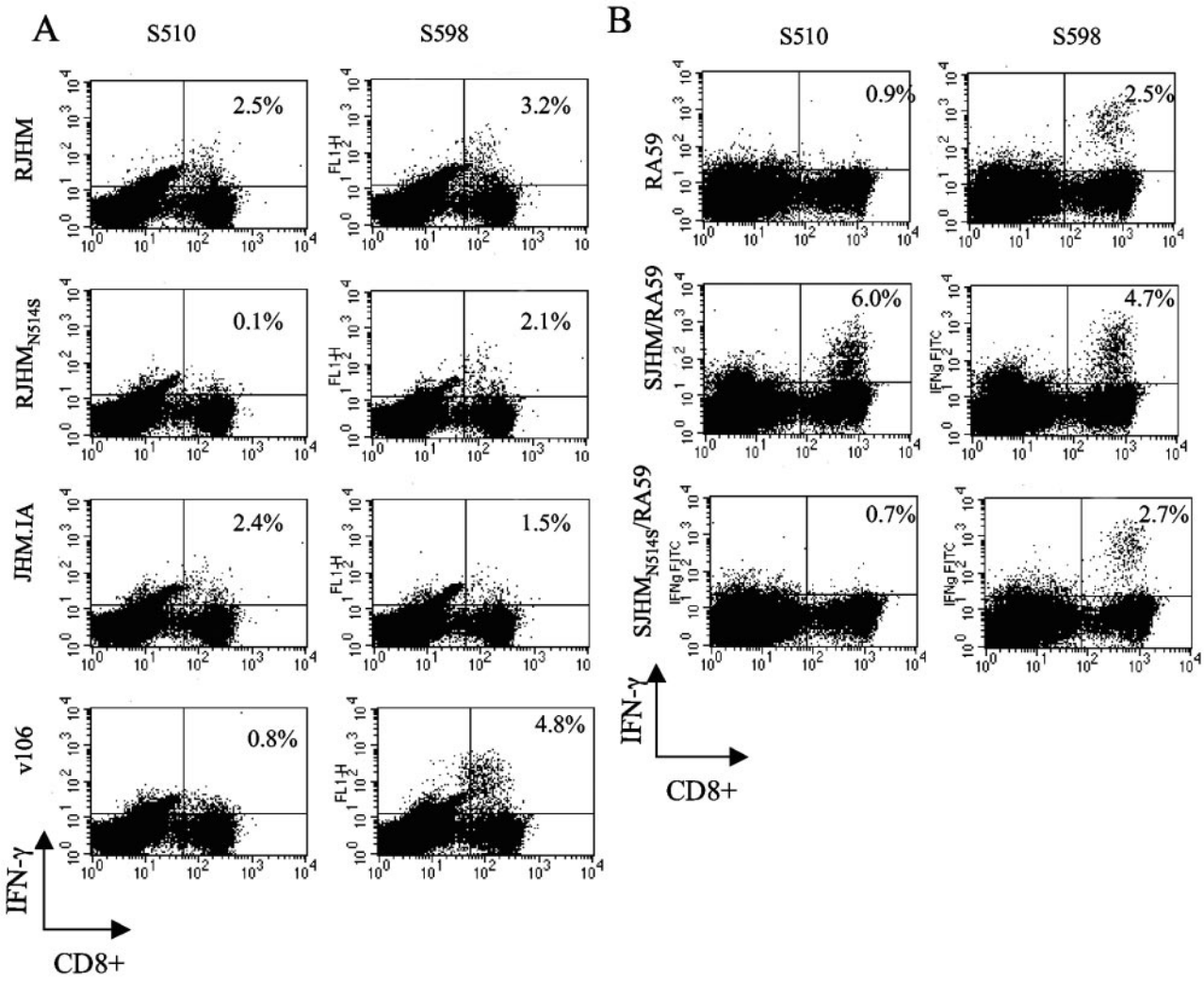

FIG. 2. CD8$^+$ T-cell response following infection with recombinant viruses encoding wild-type JHM spike or mutant spike with the N514S substitution. (A) Mice infected with 10 PFU of either RJHM, RJHM$_{N514S}$, JHM, or v106 were sacrificed at day 6 postinfection and their spleens were removed. Splenocytes were harvested and incubated with specific peptides, as indicated above each column, followed by surface and intracellular staining for CD8 and IFN-γ (see Materials and Methods). (B) Splenocytes were isolated, at day 7 postinfection, from mice infected with 10 PFU of RA59, SJHM/RA59, or SJHM$_{N514S}$/RA59 and incubated with specific peptides as indicated. Intracellular IFN-γ secretion in response to peptide stimulation was analyzed by flow cytometry. The percent of IFN-γ- and CD8-positive T cells is represented in the upper right quadrant. A representative animal for each virus is shown from two separate experiments, each including three animals per virus.

cellular receptor (19) which likely contributes to the extreme neurovirulence of viruses expressing the JHM spike. Interestingly, while introduction of the N514S mutation did not abrogate the ability to spread independent of receptor (data not shown), it resulted in attenuation of SJHM$_{N514S}$/RA59 (Table 1). The LD$_{50}$ value of SJHM$_{N514S}$/RA59 was about sixfold higher than that of SJHM/RA59 in B6 mice. Thus, in both viral genetic backgrounds, the N514S mutation did not confer an advantage in B6 mice.

We hypothesized that attenuation due to the N514S substitution was attributable to a difference in the immune response elicited by the virus, with the possibility that the CD8$^+$ T-cell response directed at the S510 epitope was pathogenic or attenuation was due to a decrease in virus spread and/or replication as a result of a functional change of the spike proteins due to the N514S mutation. In order to address this LD$_{50}$

TABLE 1. LD$_{50}$ values of recombinant viruses with or without the targeted disruption of the S510 epitope in C57BL/6 and BALB/c mice

Virus	Epitope[a](H-2D^b)	LD$_{50}$ (CFU) (log LD$_{50}$)	
		C57BL/6 (*H-2D^b*)	BALB/c (*H-2D^d*)
RJHM	CSLW**N**GPHL	4.5 (0.65)	5 (0.7)
RJHM$_{N514S}$	CSLW**S**GPHL	16 (1.2)	16 (1.2)
JHM	CSLW**N**GPHL	5 (0.7)	5 (0.7)
v106	CSLW**S**GPHL	16 (1.2)	16 (1.2)
RA59	Not present[b]	3×10^3 (3.5)	2×10^4 (4.3)
SJHM/RA59	CSLW**N**GPHL	5 (0.7)	6.3 (0.8)
SJHM$_{N514S}$/RA59	CSLW**S**GPHL	32 (1.5)	8×10^2 (2.9)

[a] The sequence of the S510 epitope is indicated with the anchor amino acid bold and underlined to indicate the presence or absence of the mutation in each virus.

[b] A59 contains a deletion within the hypervariable region of spike which encompasses the S510 epitope.

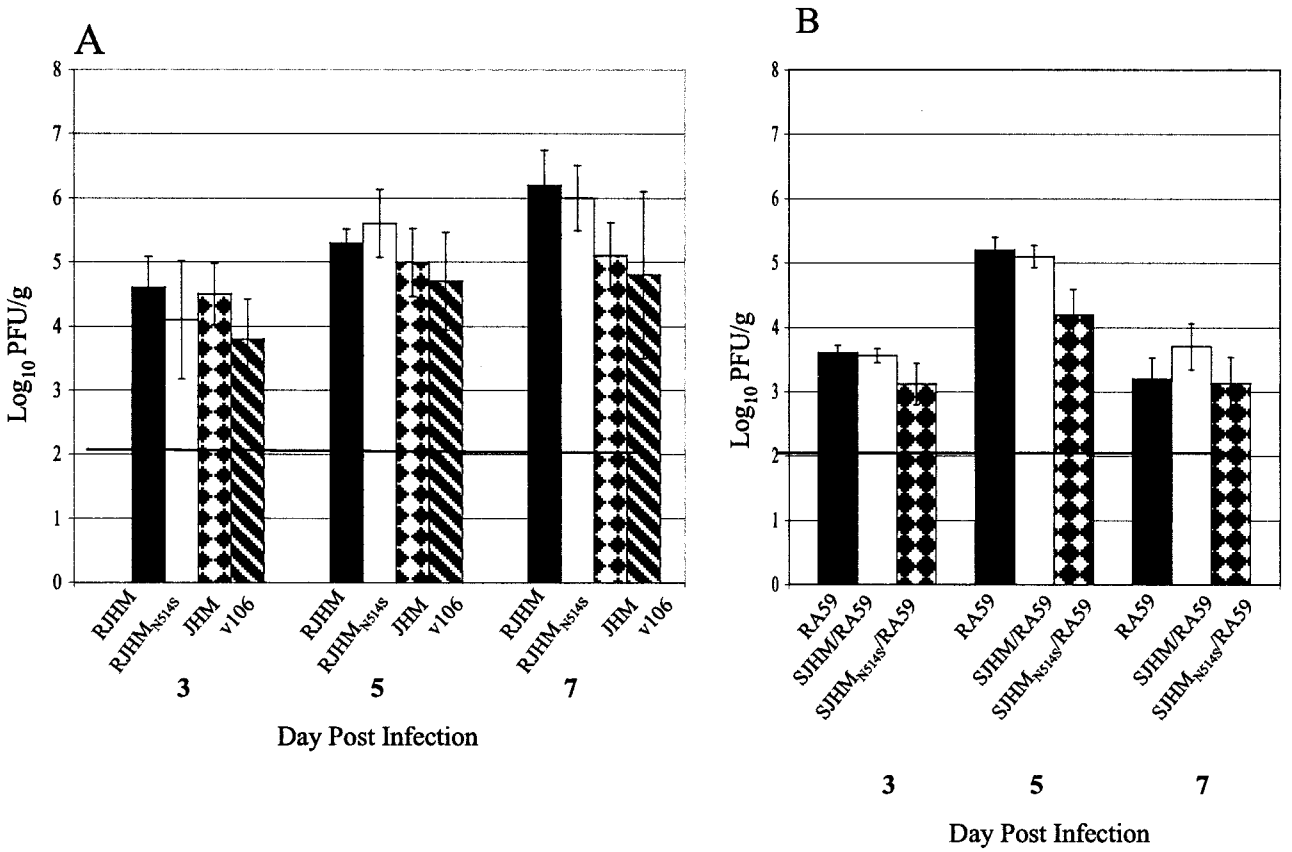

FIG. 3. Replication of epitope mutants in B6 mice. B6 mice were infected with 10 PFU of virus and sacrificed days on days 3, 5,7, 12, and 28 p.i.; brains were homogenized and infectious virus was titered on L2 cells. Viral titers are shown for days 3, 5, and 7. (Titers were below the limit of detection on days 12 and 28 and are not shown.) The data were pooled from two experiments that included five mice per virus per time point, except as indicated. (A) Mice were infected with JHM background viruses RJHM (black bar); $RJHM_{N514S}$ (white bar); JHM.IA (gray bar); v106 (striped bar). By 7 days p.i. RJHM and JHM.IA infections resulted in nearly uniform death and the survivors ($n = 2$) were moribund. Infection with $RJHM_{N514S}$ and v106 resulted in more survivors at day 7 p.i. ($n = 4$ and $n = 5$, respectively). There were no significant differences in titer among the viruses at any of the time points. (B) Mice were infected with A59 background viruses $SJHM_{N514S}$/RA59 (checked bar); SJHM/RA59 (open bar); RA59 (closed bar). At day 5, mice infected with $SJHM_{N514S}$/RA59 have significantly lower titers than mice infected with SJHM/RA59 or RA59 (Mann-Whitney U test, $P < 0.05$).

values were determined in BALB/c mice, which have an H-2^d haplotype and, thus, do not recognize the H-2^b-restricted S510 or S598 epitope. Therefore, it was expected that the wild-type and N514S mutant viruses should have the same LD_{50} value in BALB/c mice if attenuation of the N514S mutants were due to the removal of a pathogenic $CD8^+$ T-cell immune response directed at the S510 epitope.

The LD_{50} values for the epitope mutants in the JHM background were nearly identical in BALB/c and B6 mice (Table 1). We observed that wild-type RA59 was less virulent in BALB/c than in B6 mice, corresponding to an approximately sixfold increase in the LD_{50}. The contribution of the JHM spike to neurovirulence is highlighted by the fact that SJHM/RA59 exhibits dramatically increased virulence compared to RA59 in BALB/c mice, resulting in an LD_{50} value of approximately 5 PFU. Thus, the JHM spike in either the A59 or JHM background confers greatly increased neurovirulence in both B6 mice and BALB/c mice. However, introduction of the N514S mutation severely attenuated $SJHM_{N514S}$/RA59 in BALB/c mice, corresponding to a 25-fold increase in the LD_{50} compared to its LD_{50} in B6 mice (Table 1). The dramatic reduction in virulence observed for $SJHM_{N514S}$/RA59 in BALB/c mice likely reflects a combination of a functional defect in the spike protein as a result of the N514S mutation, since there is only a single amino acid change, and an overall altered immune response to the RA59 background (suggested by the difference observed with RA59 infection of B6 versus BALB/c mice).

Virus replication in vivo. In order to better establish whether the S510 epitope is required for clearance, mice were infected with 10 PFU of virus intracranially and virus replication was quantified. Brains were harvested at different times p.i. and virus from brain homogenates was titered on murine L2 cells. There were no significant differences in virus titers in the brains of RJHM- and $RJHM_{N514S}$-infected mice on all days tested postinfection (Fig. 3A). There were also no differences in titers obtained from JHM- and v106-infected mice at the time points examined. However, by day 7 p.i. most animals had succumbed to the infection and viral titers in the remaining moribund survivors were high, suggesting inefficient control of virus replication for all viruses regardless of the inactivation of the S510 epitope via the N514S mutation (Fig. 3A).

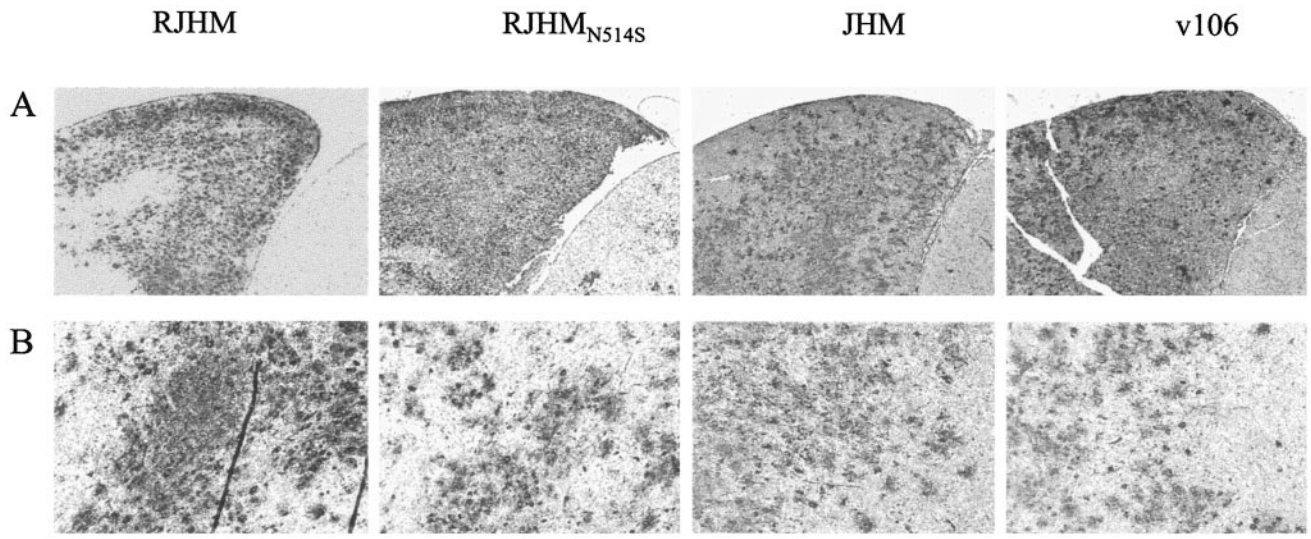

FIG. 4. Viral antigen spread in brains from B6 mice infected with JHM background viruses. Mice were infected with 10 PFU of each virus and sacrificed on day 5; sagittal brain sections, prepared as described in Materials and Methods, were stained with a monoclonal antibody directed against the nucleocapsid protein. Sections represent samples from two separate experiments with at least four animals per group. Represented are two regions that showed consistent antigen staining in all animals examined: the subiculum (A) and the midbrain (B). Magnification, 60×.

Thus, the loss of a functional S510 epitope did not diminish virus clearance or alter the kinetics of virus clearance.

RA59 and SJHM/RA59 infection produced similar titers on all days examined. However, virus titers in SJHM$_{N514S}$/RA59 infected animals were slightly lower than SJHM/RA59 and RA59-infected animals and this difference was statistically significant on day 5 p.i., the peak of virus titers (Fig. 3B). Importantly, virus with or without the N514S mutation was cleared with similar kinetics (Fig. 3B) and infectious virus was undetectable in all survivors by day 15 p.i. (data not shown). In addition, all mice infected with 10 PFU of SJHM$_{N514S}$/RA59 uniformly survived the acute infection, whereas approximately 50% of the SJHM/RA59-infected mice had succumbed to the infection by day 12 p.i. Thus, not only was the S510 epitope not essential for clearance, but epitope mutants in the A59 background were less virulent.

The observation that the epitope mutants, in the presence of either background genes, did not demonstrate increased viral titers in the brains of infected mice suggests that the loss of the S510 epitope does not confer a replicative advantage and the CD8$^+$ T-cell response to the S598 epitope, as well as possibly other unidentified CD8$^+$ T-cell epitopes and other non-major histocompatibility complex class I-restricted mechanisms, are sufficient to mediate clearance.

Virus antigen spread in the CNS is dependent upon the genetic background and position N514 within the spike HVR. Viral titers do not always accurately describe the degree of disease or pathology induced by MHV as RA59 clearly replicates to high titers within the CNS by day 5 p.i. (Fig. 3B) but is not as virulent as RJHM (Table 1). In several previous studies, we have found that the extent of virus spread throughout the brain is a better indicator of neurovirulence (7, 44, 51). To determine viral antigen spread within the brains of infected mice, sagittal sections were examined for viral antigen by staining with a monoclonal antibody that recognizes the MHV nucleocapsid protein (N) of both A59 and JHM. Mice were infected (intracranially) with 10 PFU of each virus, brains were harvested at 3, 5, and 7 days p.i., and sections were prepared. Antigen staining was minimal in all animals on day 3 and extremely variable on day 7, with day 5 brain samples exhibiting consistent intense antigen staining for all viruses examined.

Viruses containing the background genes of JHM exhibit robust virus antigen staining in many regions of the brain on day 5 p.i. that included the following: olfactory bulbs, basal ganglia, corpus callosum, thalamus, hypothalamus, subiculum, midbrain, hippocampus, and the hindbrain, including the medulla and pons. Two regions of the brain that represent the virus antigen staining observed were the subiculum (Fig. 4A) and midbrain (Fig. 4B). There were no detectable differences in the amount of antigen present in the subiculum (Fig. 4A) between the wild-type parental viruses and their epitope escape mutants, but more intense antigen staining was observed in the midbrain of RJHM-infected animals compared to RJHM$_{N514S}$-infected animals (Fig. 4B).

Recombinant viruses containing the A59 background genes do not spread throughout the brain to the same degree as viruses containing the JHM background (43, 45). Viral antigen staining in the brains of animals infected with the A59 recombinants reveals focal regions of antigen compared to nearly confluent antigen spread as seen with the JHM recombinants. All recombinant viruses expressing the A59 background genes demonstrated viral spread to similar regions of the brain on day 5 p.i. including the olfactory bulb, basal ganglia, subiculum, medulla, pons, and thalamus. The degree of spread within the regions of the brain, reflected by the level of antigen spread, was dependent upon the spike, with the JHM spike dictating enhanced spread (43). Viral antigen staining was much greater in SJHM/RA59-infected animals compared to RA59-infected animals, as previously reported (43). Importantly, mice infected with SJHM/RA59 displayed greater antigen staining in

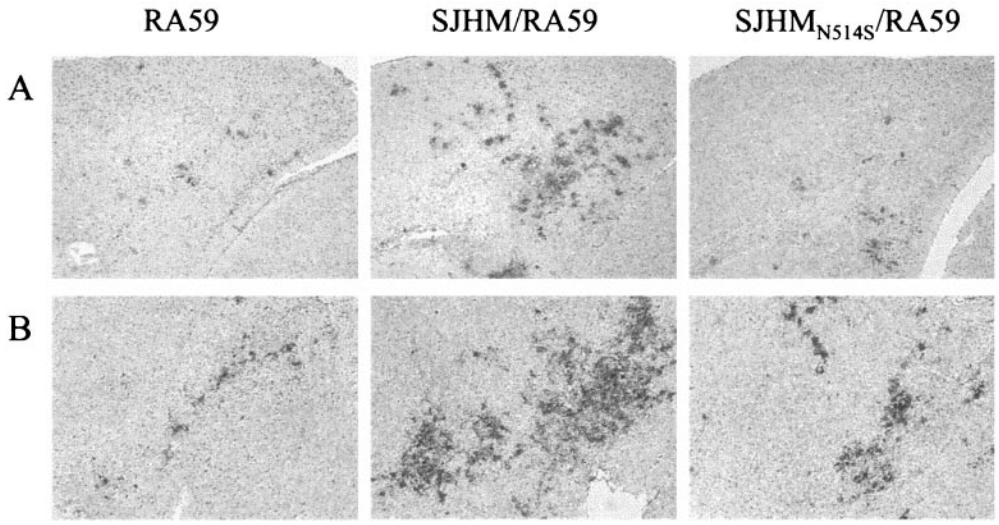

FIG. 5. Viral antigen spread in brains from B6 mice infected with the A59 background viruses. Brain sections from mice sacrificed 5 days postinfection with10 PFU of RA59, SJHM/RA59, or SJHM$_{N514S}$/RA59 were prepared and stained with a monoclonal antibody directed against the nucleocapsid protein as in Fig. 4 for immunohistochemistry for viral antigen. The regions of the brain represented are the subiculum (A) and the midbrain (B). Several sections were stained from at least four different animals from two separate experiments. Magnification, 60×.

the subiculum and midbrain compared to both RA59- and SJHM$_{N514S}$/RA59-infected animals (Fig. 5 B and C).

Interestingly, SJHM$_{N514S}$/RA59-infected animals had levels of antigen staining more similar to those of RA59 than SJHM/RA59-infected animals, with both viruses exhibiting more pronounced antigen staining in the olfactory bulb (Fig. 5A). This was surprising since the spike protein, which is the main determinant of virus spread in the brain (44), differs between SJHM$_{N514S}$/RA59 and SJHM/RA59 by only one amino acid. This suggests that the N514S mutation alters the spike protein such that it compromises its role in the virus life cycle in vivo. Furthermore, the contrast between antigen staining observed in RJHM$_{N514S}$ and SJHM$_{N514S}$/RA59-infected animals demonstrates that the background genes, other than spike, contribute significantly to the ability of virus to spread within the mouse brain.

Attenuation of SJHM$_{N514S}$/RA59 in BALB/c mice. In order to further explore why SJHM$_{N514S}$/RA59 was notably attenuated in BALB/c mice, in vivo virus replication and antigen spread were examined after infection with10 PFU of RA59, SJHM/RA59, or SJHM$_{N514S}$/RA59. Despite replicating to high titers in BALB/c mice (Fig. 6A), antigen staining revealed that RA59 did not spread throughout the CNS, likely explaining the high LD_{50} for this virus (Fig. 6B). In fact, antigen was primarily detected in the olfactory bulb (data not shown) and, furthermore, only minimal staining was observed in the midbrain (Fig. 6B) and little to no antigen was observed in any other region of the brain. However, SJHM/RA59-infected BALB/c mice exhibited virus antigen staining in similar regions of the brain in both B6 mice and BALB/c mice. While the introduction of the JHM spike can enable A59 to spread more extensively within the brains of both B6 and BALB/c mice, the N514S mutation can clearly abrogate this phenotype. Interestingly, reduced virus replication and virus spread as a result of the N514S mutation in the context of the A59 background genes is more dramatic in the BALB/c background.

Elimination of the S510 epitope does not result in virus persistence or increased demyelination. Introduction of the N514S mutation was not sufficient to establish a persistent infection within the CNS of 4-week-old mice. Infectious virus was not detected in brains or spinal cords on day 12 or 28 p.i. in any of the surviving animals analyzed (data not shown). Since the JHM recombinants were highly lethal at a dose of 10 PFU, resulting in nearly uniform death within the first week of infection, it was only possible to study the viruses with A59 background genes during the demyelinating stage of disease (4 weeks p.i.).

To evaluate the role of the N514S mutation in demyelination, B6 mice were infected with a low dose (5 to 10 PFU) and a high dose (2,500 PFU for RA59 and 100 PFU for SJHM/RA59 and SJHMN514S/RA59) of each virus. Spinal cords were removed and evaluated 30 days p.i. for myelin loss. Spinal cord cross sections, spanning the cervical to sacral regions, were stained with the myelin-specific dye luxol fast blue. The percent demyelination was determined by counting quadrants that contained demyelinated lesions, and roughly 40 to 80 quadrants were counted per mouse.

Despite displaying a highly virulent phenotype during the acute infection, SJHM/RA59 did not induce significant demyelination during the chronic infection at either dose (Fig. 7). Infection with SJHM$_{N514S}$/RA59 resulted in slightly but not statistically significantly higher levels of demyelination compared to SJHM/RA59 at both doses (Fig. 7). However, RA59 induced demyelination in a dose-dependent manner; a low dose resulted in 38.7% of spinal cord quadrants exhibiting myelin loss and at the high dose 62.4% of the spinal cord quadrants exhibited demyelination. This was significantly more than the viruses expressing the JHM spike, either wild type or encoding N514S ($P < 0.001$) (Fig. 7). Thus, RA59 induced only mild acute encephalitis accompanied by minimal virus spread during the acute phase of infection, but severe chronic demyelination occurred after infectious virus was cleared. Interest-

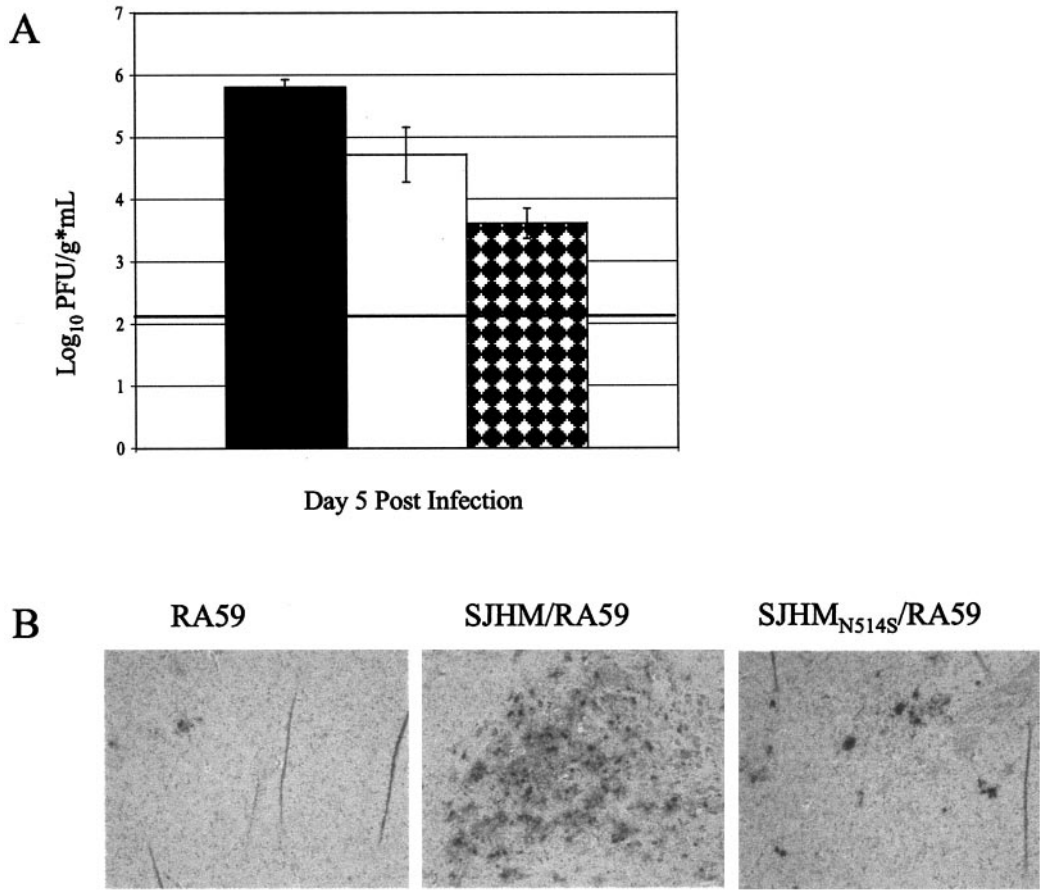

FIG. 6. Replication and antigen spread in BALB/c mice infected with A59 background viruses. BALB/c mice infected with 10 PFU of RA59, SJHM/RA59, or $SJHM_{N514S}$/RA59 were sacrificed at day 5 postinfection. (A) Half of each brain was homogenized and titered for infectious virus as described in Materials and Methods. RA59 (solid bar) and SJHM/RA59 (open bar) demonstrated significantly higher titers than $SJHM_{N514S}$/RA59 (checked bar) (Mann-Whitney U test, $P < 0.01$). (B) Half of each brain was sectioned sagitally and stained for viral antigen as described in Materials and Methods. Representative images are shown of the midbrain from a representative animal for each virus. Magnification, 60×.

ingly, while $SJHM_{N514S}$/RA59 demonstrated reduced neurovirulence and virus antigen spread (Fig. 5) compared to SJHM/RA59 and more closely resembled RA59 during the acute infection, it diverged from RA59 in its ability to cause demyelination during the chronic phase of infection. Thus, inactivation of the immunodominant epitope alone is not sufficient to confer a highly demyelinating phenotype.

DISCUSSION

Virus evasion of the $CD8^+$ T-cell response has been identified as a factor in the pathogenesis of several human diseases caused by viruses. including human immunodeficiency virus and hepatitis C and B virus-induced hepatitis (1, 6, 22, 25, 37, 52) as well as in several animal systems, including lymphocytic choriomeningitis virus and MHV (30, 31, 41). Understanding the role of epitope escape in viral pathogenesis relies on the ability to evaluate the evolution of virus mutations while simultaneously determining their effect on pathogenesis. This is difficult to analyze in a natural infection, where the origin of the infectious virus may be unknown with respect to both time and genetic composition. Thus, animal models in which cytotoxic T-lymphocyte escape occurs are essential in addressing the importance of epitope escape in pathogenesis. In the present study a single-amino-acid substitution, which abrogates the immunodominant S510 epitope, was introduced into wild-type JHM spike and, contrary to previous observations in suckling mice (42), this did not increase virulence, viral growth kinetics, or spread in vivo.

The findings of this study highlight two important features regarding fitness and virulence of the murine coronavirus. First, inactivation of an immunodominant epitope does not necessarily confer increased replication or a more virulent phenotype or establish a persistent infection in the CNS, and, second, both the hypervariable region within the spike protein and genes other than the spike (referred to here as background genes) contribute significantly to virulence. We show here that a single-amino-acid substitution within the hypervariable region of spike was capable of altering virus spread and virulence, with more dramatic attenuation observed in infections of B6 and BALB/c mice with virus recombinants containing background genes derived from the A59 strain of MHV.

The spike glycoprotein plays a major role in coronavirus infections as the critical determinant of virus entry into cells,

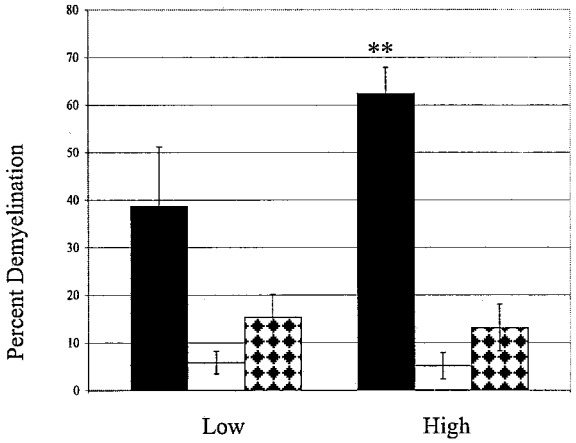

FIG. 7. Demyelination following infection of B6 mice with A59 background viruses. Demyelination was examined in B6 mice infected with RA59 (solid bar), SJHM/RA59 (open bar), and $SJHM_{N514S}$/RA59 (checked bar). Animals were infected with a low dose (5 PFU of RA59 or 10 PFU of SJHM/RA59 and $SJHM_{N514S}$/RA59) and a high dose (2,500 PFU of RA59 and 100 PFU of SJHM/RA59 and $SJHM_{N514S}$/RA59) of virus and spinal cords were removed day 30 p.i., processed, and stained with luxol fast blue as described in Materials and Methods. Sections were examined for demyelination by counting demyelinated quadrants, at least 40 quadrants per mouse. The scores shown are the averages from 5 to 10 mice per virus per dose. At both doses, RA59 induces a greater percentage of demyelination than the viruses containing the JHM spike. At the high dose this was significantly different (**, $P < 0.001$, two-tailed t test). SJHM/RA59 and $SJHM_{N514S}$/RA59 exhibit similar percentages of demyelination at both low and high doses.

mediating both attachment and fusion, and also as a primary antigenic determinant eliciting both T- and B-cell responses (5, 9, 17). The spike is a type 1 viral fusion protein, and the structure of the fusion core of MHV and severe acute respiratory syndrome coronavirus has recently been described to be similar to that of gp160 of human immunodeficiency virus (50, 53). The hypervariable region, a domain of high sequence variation within S1 of MHV, is defined to be between amino acids 400 and 600 of the JHM spike (39, 40). It was first identified in variants of JHM selected for their resistance to neutralizing monoclonal antibodies. The precise function of the hypervariable region has not been defined, but there is a clear relationship between deletions or mutations within the hypervariable region and altered viral fusion and neurovirulence (20).

Using targeted recombination, the hypervariable region of JHM was replaced with the hypervariable region from the A59 strain, which contains a 52-amino-acid deletion; this completely diminished the neurovirulent phenotype of JHM (45). Our data suggest that the effect of a single-amino-acid mutation in the hypervariable region outweighs any advantage the virus may gain by the elimination of an immunodominant CD8 T-cell epitope. Interestingly, mutations have never been reported in the subdominant H-$2K^b$-restricted epitope in the spike at positions S598 to 605. Furthermore, efforts in our laboratory to select virus recombinants with mutations in this epitope were unsuccessful, and we were unable to recover viable virus recombinants and thus we were unable to study its role in virus clearance (unpublished data). This suggests that this region of spike, just downstream of the hypervariable region domain, is essential for replication. While the hypervariable region can withstand mutation, it is an important determinant of virulence, as changes in this region attenuate virulence in animals while having no measurable effects on replication in vitro (45).

While it has been well accepted that spike glycoprotein plays a vital role in MHV pathogenesis (43), more recently, the role of the less well characterized background genes in both tropism and virulence has also been observed. Viral genes derived from JHM, other than spike, play an important role in abrogating the ability of a recombinant A59/JHM chimeric virus to induce hepatitis (36). Preliminary data suggest that this is most likely not a property of the JHM replicase but rather the other structural genes encoded at the 3′ end of the genome (S. Navas and S. R. Weiss, unpublished data). The role of background genes in virulence is further demonstrated by the more pronounced attenuation due to the N514S mutation in the A59 background compared to the JHM background.

We observed a slight difference in the LD_{50} values of $SJHM_{N514S}$/RA59 and $RJHM_{N514S}$ in B6 mice and a more dramatic difference in BALB/c mice (Table 1). The rate at which animals succumb to the infection is also very different for these two viruses. In BALB/c mice, $RJHM_{N514S}$ is still highly virulent, but $SJHM_{N514S}$/RA59 has dramatically reduced virulence. Attenuation of $SJHM_{N514S}$/RA59 is due to both the N514S mutation, as SJHM/RA59 is still highly virulent in BALB/c mice as well as the background genes of A59, since the epitope mutant with the JHM background, $RJHM_{N514S,}$ is highly lethal. It is presently not clear why both RA59 and $SJHM_{N514S}$/RA59 are more attenuated in BALB/c mice than in B6 mice. B6 and BALB/c mice have different haplotypes, and thus, they recognize different epitopes which may alter the ability to clear virus. However, it is also known that BALB/c mice easily induce a Th2 response, likely due to a reduction in interleukin-12 receptor expression, and this likely influences the overall immune response (26). It is interesting that a stronger Th2 response may be more protective against RA59 infection, but clearly shows no protection against an infection with viruses containing the JHM background genes.

The ability of MHV to spread to particular regions of the brain has been shown to be an important determinant of neurovirulence (44, 51). Here we observe that a single-amino-acid mutation in the spike glycoprotein is sufficient to reduce virus spread, as determined by virus antigen staining, which reduces virulence. We observe a role for both the viral background and the spike. Mice infected with viruses with the JHM background genes exhibited viral antigen throughout the brain by day 5 p.i., and we observe high levels of antigen in the hippocampus, medulla, and pons which likely contributes to the very early death mediated by these viruses.

When the JHM spike is introduced into the A59 background, the recombinant is nearly as virulent as wild-type JHM (although mice begin to die at a later time postinfection). However, while antigen staining observed in SJHM/RA59-infected animals is greater than with RA59, it is never as robust as with wild-type JHM. Thus, there are additional contributions of the JHM background that allow rapid virus spread. This also suggests that there is threshold for virus spread, such that even if SJHM/RA59 cannot induce as robust a virus spread as RJHM, it can infect particular regions well enough to

cause nearly uniform death. Interestingly, RA59 spreads and replicates well in the neurons of the olfactory bulb, accounting for the high titers obtained from mice, but it does not spread well in neurons in other regions of the brain. The inability of RA59 to spread well in neurons throughout the brain is likely the main determinant of its neuroattenuated phenotype. Similarly, in a previous study from this laboratory (51), attenuated mutants of SJHM/RA59 were restricted in spread to the olfactory bulb but exhibited wild-type levels of infectious virus in the brain. This was explained by the finding that for both mutant and parental viruses, most of the infectious virus was in the olfactory bulb. In the present study, it was particularly interesting that the N514S mutation induced attenuation that largely depended upon the viral background genes.

Animals infected with viruses that contain background genes derived from JHM rarely survived long enough to be analyzed for demyelination, while recombinants with A59 background genes were slightly attenuated permitting analysis at the later time points that are required in order to observe demyelination. Demyelination was much reduced in recombinants containing the JHM spike, with or without the N514S mutation, in comparison to RA59. One possibility to account for this is that the JHM spike induces extensive cellular damage, compared to the A59 spike, such that only those mice that experienced very limited virus spread would survive the infection. Virus spread to the spinal cord is essential for the demyelination process (33), and thus, the survivors of JHM infection (which likely did not experience extensive virus spread) subsequently do not develop significant demyelination. It is also possible the low levels of demyelination are due to the propensity of viruses containing the JHM spike to preferentially infect neurons rather than glia in the spinal cord; variants of JHM that contain deletions within S1 of spike have altered tropism for glial cell types and have been shown to be both less virulent and more demyelinating (2, 16, 18).

In contrast to observations in suckling mice, epitope mutations have not been observed in virus isolated from adult mice infected with MHV that go on to develop chronic demyelination and paralysis (3). Furthermore, using a vaccination strategy to protect against MHV, epitope escape was observed by day 5 p.i., but the immune evasion did not abrogate protection. Mice were first immunized with a recombinant strain of *Listeria monocytogenes* expressing the gp33 epitope, from the glycoprotein of lymphocytic choriomeningitis virus, and then infected with a recombinant strain of MHV expressing the gp33 epitope as a fusion to a nonessential protein. Epitope escape occurred readily, likely due to the fact that the gp33 epitope was not essential to virus fitness, however, escape from the gp33-specific immune response did not prevent effective clearance or permit virus persistence. Thus, there appear to be unique features of the suckling model that may create an ideal setting in which escape mutants can arise and also for these epitope escape mutants to play a role in pathogenesis. While passive antibody is protective against acute encephalitis in the suckling mice, it is likely that virus clearance is not achieved. Inefficient clearance of virus in the suckling mouse model may alter the immune response as well as the state in which virus persists, thus creating conditions optimal for the reemergence of infectious virus.

In addition to the role of $CD8^+$ T cells in virus clearance, B cells also contribute to protection from viral infections in the CNS. The anti-MHV antibody response can prevent acute encephalitis in the suckling model and, more recently, it has been demonstrated that the prevention of virus recrudence in the MHV-infected CNS is largely dependent upon the antibody response (11, 32, 34, 46). Mice that have B cells but are unable to secrete antibodies clear virus with normal kinetics, but virus begins to reemerge two weeks postinfection (32). This is an organ-dependent phenomenon, as infection with the dualtropic A59 strain of MHV revealed that reemergence of virus in the liver does not occur in the absence of antibodies (34). Dandekar et al. recently described the role of MHV-specific antibodies in preventing persistent infection in the suckling model of JHM-induced demyelination. While a large percentage of suckling B6 mice developed a persistent infection, infection of BALB/b mice, which are also *H-2*^{b} and recognize the same $CD8^+$ epitopes, did not result in virus persistence. Their studies also revealed that BALB/b mice had 25-fold more MHV-specific antibody-secreting cells within the CNS. Interestingly, administration of anti-MHV neutralizing antibodies suppressed the emergence of epitope escape mutants in that model (11).

The finding that abrogation of the immunodominant epitope is not sufficient to prevent virus clearance of MHV from the CNS suggests that the S598 epitope and possibly other, unidentified epitopes as well as non-major histocompatibility complex class I-restricted mechanisms, are important for clearance. The data presented here clearly show that there is a very fine balance between the escape from an immune response and maintenance of virus fitness; we suggest that this balance determines the level of pathogenesis. Thus, a small change in the spike protein is able to greatly reduce virulence. Finally, the difference in the effects of epitope mutations in B6 versus BALB/c mice suggests that other host immune effectors are important for the clearance of infectious virus and the determination of the final outcome of infection.

ACKNOWLEDGMENTS

This work was supported by NIH grants AI 47800, AI 60021 (S.R.W.), AI 45025 (H.S.), and K.C.M. was supported in part by NIH training grant AI 007324.

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