JOURNAL OF VIROLOGY, Dec. 2005, p. 15064–15073 Vol. 79, No. 24
0022-538X/05/$08.00+0 doi:10.1128/JVI.79.24.15064–15073.2005

Expression of Hemagglutinin Esterase Protein from Recombinant Mouse Hepatitis Virus Enhances Neurovirulence

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Received 20 June 2005/Accepted 21 September 2005

Murine hepatitis virus (MHV) infection provides a model system for the study of hepatitis, acute encephalitis, and chronic demyelinating disease. The spike glycoprotein, S, which mediates receptor binding and membrane fusion, plays a critical role in MHV pathogenesis. However, viral proteins other than S also contribute to pathogenicity. The JHM strain of MHV is highly neurovirulent and expresses a second spike glycoprotein, the hemagglutinin esterase (HE), which is not produced by MHV-A59, a hepatotropic but only mildly neurovirulent strain. To investigate a possible role for HE in MHV-induced neurovirulence, isogenic recombinant MHV-A59 viruses were generated that produced either (i) the wild-type protein, (ii) an enzymatically inactive HE protein, or (iii) no HE at all (A. Lissenberg, M. M. Vrolijk, A. L. W. van Vliet, M. A. Langereis, J. D. F. de Groot-Mijnes, P. J. M. Rottier, and R. J. de Groot, J. Virol. 79:15054–15063, 2005 [accompanying paper]). A second, mirror set of recombinant viruses was constructed in which, in addition, the MHV-A59 S gene had been replaced with that from MHV-JHM. The expression of HE in combination with A59 S did not affect the tropism, pathogenicity, or spread of the virus in vivo. However, in combination with JHM S, the expression of HE, regardless of whether it retained esterase activity or not, resulted in increased viral spread within the central nervous system and in increased neurovirulence. Our findings suggest that the properties of S receptor utilization and/or fusogenicity mainly determine organ and host cell tropism but that HE enhances the efficiency of infection and promotes viral dissemination, at least in some tissues, presumably by serving as a second receptor-binding protein.

Coronaviruses, which are enveloped positive-strand RNA viruses of mammals and birds, are pathogens of medical and veterinary relevance. Interest in their biology and pathogenic properties has increased considerably with the recent identification of three new human coronavirus (HCoV) species, namely, severe acute respiratory syndrome HCoV (SARS-HCoV) (16), HCoV-NL63 (10, 51), and HCoV-HKU1 (56). Murine hepatitis virus (MHV) infection of mice provides a well-established animal model for coronavirus-induced disease (20). Depending on the strain, inoculation route, and dose, MHV produces hepatitis, acute encephalitis, or chronic demyelination. Adding to the strength of the model, MHV is amenable to reverse genetics.

MHV, a group II coronavirus, has a 32-kb genome and encodes at least four structural proteins: the nucleocapsid protein (N), the membrane glycoprotein (M), the spike glycoprotein (S), and the small envelope protein (E). The S protein plays a pivotal role during infection. It binds to specific host cell receptors, isoforms of the murine carcinoembryonic antigen cell adhesion molecule 1a (mCEACAM) (9), and mediates fusion between the viral envelope and the plasma membrane as well as between the membranes of infected and adjacent noninfected cells (for reviews, see references 5 and 12). A fifth structural protein, the hemagglutinin esterase (HE), is an accessory, 60- to 70-kDa type I envelope glycoprotein unique to group II coronaviruses (3); it is also found, among others, in bovine coronavirus (BCoV) and in HCoV strains OC43 and HKU1 (56). Disulfide-bonded HE dimers (or multimers thereof) are incorporated into virions and, in electron micrographs, appear as small surface projections of 5 to 7 nm which are clearly distinct from the prominent 20-nm peplomers comprised of S (3, 4, 6, 23a, 47).

Although HE is absent from coronaviruses belonging to groups I and III as well as from SARS-HCoV (26, 39), related proteins that are 30% identical to HE do occur in toroviruses (6, 8, 45, 46) and in influenza C virus (24), in the latter case as the N-terminal subunit of the HE fusion protein (HEF). HEF is a multifunctional protein, as (i) it binds specifically to 5-*N*-acetyl-9-*O*-acetyl-*N*-neuraminic acid (Neu5,9Ac$_2$) receptor determinants, (ii) it carries sialate-9-*O*-acetylesterase activity, acting as a receptor-destroying enzyme (RDE), and (iii) it mediates pH-dependent membrane fusion activity (14).

Unlike HEF, the coronavirus HE does not mediate fusion and is not required for viral entry into and replication in cultured cells (11, 35). In fact, in many MHV laboratory strains, the HE gene is inactivated (59), apparently as an artifact resulting from adaptation to propagation in vitro (23a). MHV-A59 does not produce mRNA2b, the mRNA for HE, because of a mutation in the transcription-regulating sequence, and in addition, its HE gene is interrupted by a nonsense mutation at codon 15 (24, 43). Nevertheless, MHV-A59 grows

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to very high titers in vitro, even more efficiently than some strains such as MHV-JHM, -DVIM, and -S that have retained HE expression.

Apparently, HE is also dispensable in vivo. MHV-A59, though significantly less neurovirulent than MHV-JHM, still causes hepatitis and encephalitis in experimentally infected mice (28, 33). Additionally, MHV-2, a highly hepatotropic strain, is also unable to express HE (7). Furthermore, Lai and coworkers reported that in MHV-JHM-infected mice and rats, viral variants defective for HE accumulate in the brain and spinal cord (19, 60). However, in another study from the same group, greater mortality and tropism in neurons were associated with an MHV-JHM variant that expressed an abundant amount of HE than with a variant that expressed less HE (58). Taguchi et al. (49) found that HE-expressing variants of MHV-JHM are selected for during propagation in cultured neural rat cells. Also, HE expression seems to be maintained in the field. During the divergence of murine coronaviruses, HE genes have not been lost or inactivated; rather, they have been exchanged via homologous recombination, in one case with a hitherto unidentified group II coronavirus as a donor (44). Apparently, during natural MHV infection, the expression of HE provides a selective advantage. Its precise function, however, is poorly understood and subject to debate.

In analogy with HEF, coronavirus HEs might combine receptor-binding and RDE activities and mediate reversible virus attachment to sialic acid (Sia) receptor determinants, thus assisting S during entry. Indeed, coronavirus HEs have been established unambiguously as sialate-*O*-acetylesterases, and for all group 2 coronaviruses studied so far, the substrate preference of HE matches the Sia receptor specificity of the virions. For instance, BCoV, HCoV-OC43, and MHV-DVIM specifically bind to Neu5,9Ac_2 moieties (41), and in accordance, encode HEs with sialate-9-*O*-acetylesterase activity (44, 52, 53). Likewise, MHV strains S and JHM bind to 5-*N*-acetyl-4-*O*-acetylneuraminic acid (Neu4,5Ac_2) receptors and encode HEs with sialate-4-*O*-acetylesterase activity (37, 57). However, the direct involvement of HE in virion attachment has been called into question. Herrler and coworkers (17, 41) have argued that in the case of BCoV and HCoV-OC43, binding to Sia receptor determinants is mediated predominantly by the S protein. Wurzer et al. (57) have suggested that the same might be true for MHV, based on the observation that an antiserum raised against A59, and hence devoid of antibodies against HE, inhibited the binding of MHV-S to Sia in solid-phase assays. Yet, in support of a role in receptor binding, HE proteins are Sia-specific lectins, as demonstrated by hemagglutination and/or hemadsorption assays (31, 42, 61). Moreover, in the case of MHV-DVIM, hemagglutinating activity was assigned explicitly to HE and not to S (47; M. A. Langereis, A. L. W. van Vliet, and R. J. de Groot, unpublished results). Clearly, the role of HE during infection requires further investigation. Given that HE expression is nonessential in vitro and even detrimental to propagation in some cultured cells (23a), its function should best be assessed with animal experiments.

Previously, recombinant MHVs (rMHVs) generated through targeted RNA recombination (18, 27) have helped to demonstrate the importance of S in determining both neurovirulence and tropism (29, 32). There is ample evidence, however, that viral proteins other than S contribute significantly to spread in the brain (Iacono et al., manuscript in preparation) as well as to replication and virulence in the liver (30). In order to investigate a possible role for HE in MHV-induced neurovirulence, isogenic recombinant MHV-A59 viruses were generated in which the autologous defective HE gene was replaced with that of the closely related strain MHV-S. The resulting viruses produced either the wild-type protein (HE^+), an enzymatically inactive HE protein (HE^0), or no HE at all (HE^-) (23a).

Additionally, a second set of these recombinant viruses was generated in which, in addition, the S gene of MHV-A59 was replaced with that from the highly neurovirulent strain JHM (32). The expression of HE in combination with the S protein of A59 did not affect the tropism, pathogenicity, or spread of the virus in vivo. However, in combination with the JHM S protein, the expression of an intact HE polypeptide, regardless of whether it retained esterase activity, resulted in increased viral spread within the central nervous system (CNS) and in increased neurovirulence. Our findings fit a model in which properties of S (receptor preference and/or fusogenicity) ultimately determine organ and host cell tropism but in which HE enhances the efficiency of infection and promotes viral dissemination, at least in some tissues. We hypothesize that HE may have this effect by serving as a second receptor-binding protein.

MATERIALS AND METHODS

Cells, viruses, and antibodies. All cells were maintained in Dulbecco's modified Eagle medium (Gibco BRL, Gaithersburg, MD) containing 10% heat-inactivated fetal bovine serum and 1% penicillin-streptomycin in the presence of 5% CO_2 at 37°C.

Recombinant virus fMHVΔ2A/HE (23a), which served as the acceptor virus in targeted recombination experiments, was propagated in fcwf-D cells (American Type Culture Collection). Recombinant viruses rMHV-A59S-HE^+, rMHV-A59S-HE^0, and rMHV-A59S-HE^- (23a), RA59 (32), and rMHV-JHMS-HE^+, rMHV-JHMS-HE^0, and rMHV-JHMS-HE^- (see below) were propagated and titrated by plaque assays in either 17Cl-1 cells, L-2 cells, or LR7 cells (a mouse L-cell line which stably expresses the MHV receptor) (18). Cells were routinely infected with virus suspensions diluted in phosphate-buffered saline (PBS) containing 50 μg/ml DEAE dextran. Prior to inoculation, monolayers were rinsed once with the same buffer.

The monoclonal antibody 1-16-1, directed against the MHV N protein, was provided by J. Leibowitz (Texas A&M University). Rabbit polyclonal antiserum K135, raised against MHV-A59, was described previously (40).

Selection of recombinant viruses. Recombinant viruses were generated by targeted RNA recombination (18, 32, 34), with fMHVΔ2A/HE (23a) as the acceptor virus. rMHV-A59S-HE^+ (expressing the wild-type MHV-S HE protein), rMHV-A59S-HE^0 (expressing a mutant, enzymatically inactive HE protein), and rMHV-A59S-HE^- (expressing no HE at all) contain MHV-A59 background genes; their construction and in vitro growth properties are described in detail in reference 23a.

The same procedure was used to generate a new set of isogenic recombinant viruses, which in combination with MHV-S HE (and variants thereof), produced the MHV-JHM spike protein instead of the MHV-A59 spike protein. To this end, AvrII-Sbf fragments containing the MHV-A59 gene were excised from transfer vectors pMH54HE^+, -HE^0, and -HE^- (23a) and replaced with a corresponding AvrII-SbfI fragment carrying the JHM spike gene excised from pG-MHV4-S2 (32). The resulting plasmids were designated pMH54JHMSHE^+, -HE^0, and -HE^-, respectively. Capped, synthetic RNAs transcribed from these vectors served as donors in targeted RNA recombination assays with fMHVΔ2A/HE as the acceptor. In vitro transcription, targeted recombination, and the selection and plaque purification of recombinant progeny in LR7 cells were performed as described in reference 23a. Targeted recombination experiments were performed in duplicate to allow the isolation of at least two completely independent recombinants for each of the envisaged viral mutants. The expression of HE (or the lack thereof) was confirmed enzymatically and by radio-immunoprecipitation as described by Lissenberg et al. (23a); reverse transcription-PCR amplification of the 5′- and 3′-end regions of the S gene and sequence analysis confirmed that the MHV-JHM S gene had been inserted

properly in each recombinant virus. Viral stocks were grown on 17Cl-1 cells for further studies.

Virus replication in vitro. Viral growth curves were carried out on L2 monolayers in 24-well plates. Cells were infected with the recombinant viruses at a multiplicity of infection of 1 PFU/cell and incubated at 37°C for 1 h. The inoculum was aspirated, and the cells were washed with PBS, covered with Dulbecco's modified Eagle medium–10% fetal bovine serum, and further incubated at 37°C. At the indicated time points, the cells were lysed by freeze-thawing three times, the lysates were collected and clarified by centrifugation at 3,000 rpm for 20 min in an Eppendorf 5810 centrifuge, and the viral titers were determined by plaque assays on L2 monolayers as previously described (13).

Inoculation of mice. Four-week-old male C57BL/6 mice (NCI, Bethesda, MD) were used for all experiments. The mice were anesthetized with isoflurane (IsoFlo; Abbott Laboratories, Chicago, IL). For intracranial inoculation, 25 μl of virus was injected into the left cerebral hemisphere after dilutions were made with PBS containing 0.75% bovine serum albumin. For intranasal inoculation, 10 μl of virus at the desired dilution was introduced into each nostril. Controls were inoculated with uninfected cell lysates at the same dilution.

Virulence assays. The 50% lethal dose (LD_{50}) was calculated as previously described (15). Four-week-old C57BL/6 mice were inoculated intracranially with four 10-fold serial dilutions of the recombinant viruses. In two independent experiments, five animals were used per dilution of virus. The mice were observed for a period of 21 days for morbidity and mortality. LD_{50} values were calculated by the Reed-Muench method (36). For three different experiments to determine the time to death, 10 animals were used per virus. The mice were injected intracranially with 6,000 PFU (approximately 1 LD_{50}) of virus and observed for signs of disease and mortality for a period of 21 days. The percentage of survival was calculated.

Virus replication in mice. For determinations of infectious virus titers in the brain and liver, five mice per time point were infected intracranially or intranasally with each virus. The mice were sacrificed on day 5 postinfection (p.i.) (intracranial infections) or on day 3, 5, or 7 p.i. (intranasal infections) and perfused with 10 ml sterile PBS. Their brains and livers were removed. The left half of the brain and a lobe of the liver were each placed into 2 ml of isotonic saline with 0.167% gelatin (gel saline). The organs were weighed and homogenized, and viruses were titrated by plaque assays on L2 cells (15).

Immunohistochemistry. To assess the extent of viral dissemination and histopathology, the right half of the brain and a piece of the liver from the same animals described above were fixed in 10% phosphate-buffered formalin. Formalin-fixed tissues were embedded in paraffin, sectioned, and left unstained for immunohistochemistry. Brain and liver sections from mock-infected animals were used as controls. The tissues were first deparaffinized and then rehydrated. Virus-infected cells were detected by staining for the N protein with the monoclonal antibody 1-16-1 (diluted 1:20 in PBS), employing the avidin-biotin-immunoperoxidase technique for immunohistochemistry (Vector Laboratories, CA), with diaminobenzadine tetrachloride as a substrate and hematoxylin as a counterstain. At least three sagittal sections per mouse (from at least three mice/virus) were assessed in a blinded manner.

Detection of acetylesterase activity. To test for the expression of sialate-*O*-acetylesterase, plaque assays were performed on L2 monolayers with diluted supernatants of brain and liver homogenates from mice infected with rMHV-A59S-HE^+. Cells were assayed for esterase activity as described in reference 23a by in situ pararosanilin staining with α-naphthyl acetate as a substrate (55).

Analysis of virion proteins. LR7 cells were infected with plaque-purified stocks of viruses isolated from brains and livers of mice which had been inoculated with rMHV-A59S-HE^+, rMHV-A59S-HE^0, and rMHV-A59S-HE^-. The cells were metabolically labeled with 170 μCi/μl ^{35}S in vitro cell-labeling mix (Amersham) in the presence of 1 μM HR2 fusion inhibitor (2) from 2 to 10 h postinfection. Tissue culture supernatants were cleared by sequential low-speed and high-speed centrifugation, and virus particles were affinity purified from 200 μl of clarified supernatant with antiserum K135 as described in reference 23a. Samples were analyzed by electrophoresis in sodium dodecyl sulfate-polyacrylamide gels followed by fluorography.

RESULTS

Expression of HE in the MHV-A59 background does not increase virulence, tissue virus titers, or virus spread. rMHV-A59S-HE^+, rMHV-A59S-HE^0, and rMHV-A59S-HE^- (Fig. 1) are isogenic recombinant viruses which carry the HE gene of MHV-S, or mutated derivatives thereof, in the MHV-A59 background. rMHV-A59S-HE^+ produces a wild-type HE protein, while rMHV-A59S-HE^0 expresses an enzymatically inactive form of HE in which the active-site residue Ser^{45} of the esterase (54) has been replaced by Thr. rMHV-A59S-HE^- is HE deficient: this virus carries an HE gene from which the codons for residues 101 to 104 have been deleted, creating a frameshift mutation immediately downstream of codon 33 (for a detailed description of the construction of these viruses and their in vitro growth characteristics, see reference 23a. To investigate whether HE contributes to virulence, we infected mice with the recombinant viruses and determined the LD_{50} for each one. Four-week-old C57BL/6 mice were inoculated intracranially with 10^2, 10^3, 10^4, or 10^5 PFU and observed daily for morbidity and mortality for up to 21 days, at which time LD_{50} values were calculated. As shown in Table 1, the LD_{50}s were similar for all three types of recombinant viruses and were in the same range as that of the parental virus, MHV-A59 (32).

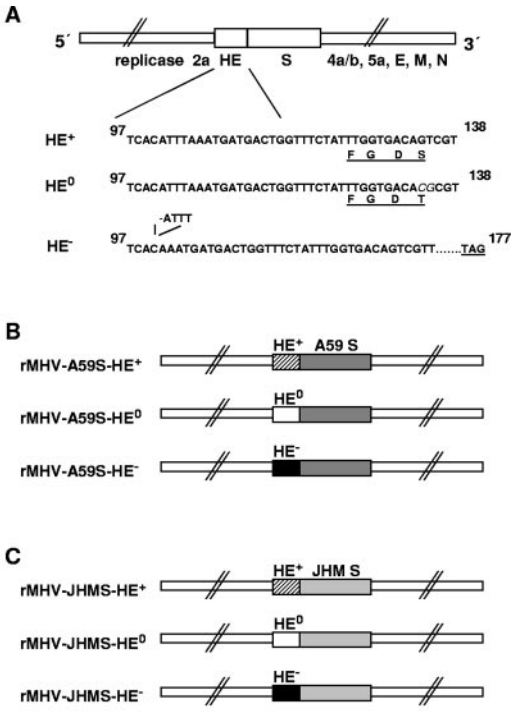

FIG. 1. Schematic representation of recombinant viruses differing in HE expression. (A) Schematic representation of MHV genome organization (top). Genes for the replicase, the structural proteins HE, S, E, M, and N, and the nonstructural proteins 2a, 4a and b, and 5a are depicted as open boxes. The bottom panel shows the relevant nucleotide and amino acid sequences of the HE variants. HE^+ designates the wild-type HE of MHV strain S; HE^0 designates an enzymatically inactive HE in which the active-site serine has been mutated to threonine; and HE^- designates a truncated HE protein (nucleotides 101 to 104 were deleted from the HE gene, causing a frameshift mutation immediately downstream of codon 33 [23a]). Recombinant viruses were generated that carry either the S gene of MHV-A59 (B; S gene indicated by a dark gray box) or that of MHV-JHM (C; S gene indicated by a light gray box). Within each set, the viruses are isogenic except for the HE gene, which is either wild type (HE^+) or encodes an inactive acetylesterase (HE^0) or a truncated HE polypeptide (HE^-). HE^+, HE^0, and HE^- are represented by striped bars, white bars, and black bars, respectively.

TABLE 1. Virulence of rMHV-HE$^+$, rMHV-HE^0, and rMHV-HE$^-$

Virus[a]	Log_{10} LD_{50}[b]
rMHV-HE$^+$	3.49
rMHV-HE$^+$	3.28
rMHV-HE^0	3.80
rMHV-HE$^-$	3.20
rMHV-HE$^-$	3.00

[a] Two independently isolated recombinants were analyzed in the case of rMHV-HE$^+$ and rMHV-HE$^-$; one recombinant was used for rMHV-HE^0.
[b] Mice were infected intracranially with four doses of virus, and LD_{50} values were calculated as described in Materials and Methods.

We next compared the survival characteristics of mice inoculated with the recombinant viruses at a dose of 6,000 PFU (~1 LD_{50}) in longitudinal experiments, during which the onset and severity of clinical signs were scored and mortality was monitored for a period of 6 weeks. All animals developed clinical signs of disease, and there were no significant differences among the groups with respect to survival time (not shown). The median times to death were 7 to 10 days. For mice which were sacrificed at 5 days p.i., i.e., at the peak of viral replication (32), similar amounts of infectious virus were found in the liver and in the brain (Fig. 2A). Apparently, the recombinant viruses did not differ significantly from each other or from RA59 (a recombinant "wild-type" A59 derivative which expresses neither the HE protein nor HE mRNA2b) with respect to the rates of viral replication in these target organs.

Since intranasal inoculation is considered a natural route of infection for MHV (48), we reasoned that HE might enhance viral dissemination and replication if the virus were to enter via this route rather than via injection directly into the brain. In support of this notion, the olfactory bulbs, the first sites of infection following intranasal inoculation, contain large amounts of virus at the peak of replication (50). We therefore inoculated C57BL/6 mice intranasally with 10,000 PFU of each recombinant virus (5,000 PFU per nostril). Animals were sacrificed on days 3, 5, and 7 p.i., and brain and liver samples were tested for infectious virus. As shown in Fig. 2B, after intranasal inoculation, rMHV-A59S-HE$^+$, -HE^0, and -HE$^-$ replicated to similar titers in the brain and liver.

Finally, we were unable to detect any effect of HE on viral dissemination in the target tissues by immunohistochemistry. Sections of brains and livers from mice which had been infected either intracranially or intranasally with rMHV-A59S-HE$^+$, -HE^0, -HE$^-$, or RA59 and sacrificed on day 5 p.i. were stained with a monoclonal antibody directed against the MHV N protein. The distributions of viral antigen within the brain, with respect to both extent and location, were similar for all recombinant viruses. Also, the livers of the infected mice showed similar extents of lesions, irrespective of the type of virus or the inoculation route (data not shown). On the basis of the combined results, we conclude that in the context of the MHV-A59 background, the expression of HE or the lack thereof does not significantly alter virulence.

HE expression in the MHV-A59 background is maintained during replication in mice. During the propagation of rMHV-A59S-HE$^+$ in cell culture, spontaneous mutants are selected for that produce defective HE proteins which are not incorporated into the virions (23a). Conceivably, the loss of HE expression in vivo might explain the lack of an obvious HE-related phenotype. To address this issue, mice infected intracranially with rMHV-A59S-HE$^+$ or rMHV-A59S-HE^0 were sacrificed on day 5 p.i., and viruses were plaque purified directly from tissue homogenates. The expression of HE and its presence in the viral envelope were assayed by immunopurification of metabolically labeled virions. Of the rMHV-A59S-HE$^+$ and -HE^0 viruses isolated from the brain, 5 of 5 and 9 of 10, respectively, contained HE in the virions, while of the viruses isolated from the liver, 3 of 5 and 9 of 10, respectively, expressed intact HE (Fig. 3). To more precisely assess the percentage of viruses that had lost HE expression and to determine whether there are differences in this respect between viral populations in livers and brains, we performed esterase staining of rMHV-A59S-HE$^+$ plaques; this approach was based on the notion that most mutations in HE that prevent incorporation into the envelope also abrogate enzymatic activity. Of the viruses from brains (n = 1,495), 97% ± 1.5% expressed a functional HE protein, while of those from livers (n = 609), 87.5% ± 1.5% were positive for O-acetylesterase; this difference was statistically significant in a t test ($P < 0.05$). However, given that after replication in vivo, the majority of rMHV-A59S-HE$^+$ and rMHV-A59S-HE^0 viruses still express HE, the lack of an obvious HE-related phenotype cannot be accounted for by the loss of this protein.

Generation and characterization of isogenic recombinant viruses differing in HE expression and expressing the JHM spike gene. MHV-A59 is highly cell adapted and displays only moderate pathogenicity. This might explain why the introduction of HE into the A59 background failed to yield increased

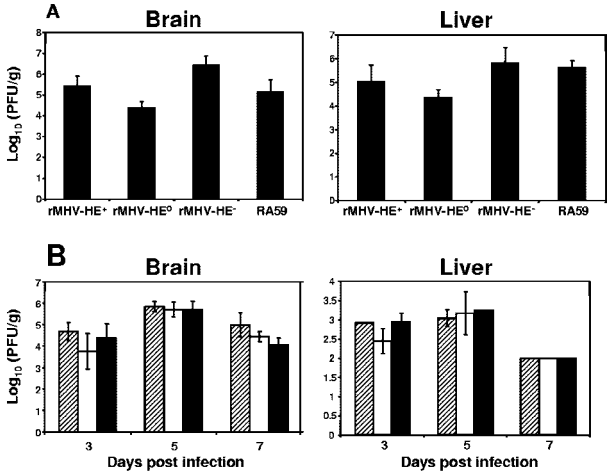

FIG. 2. Viral titers in brains and livers of mice infected with rMHV-A59S-HE$^+$, rMHV-A59S-HE^0, and rMHV-A59S-HE$^-$. Four-week-old C57BL/6 mice were inoculated by either the intracranial (A) or intranasal (B) route. Viruses were titrated from brain and liver lysates from mice sacrificed at the indicated times. Each time point represents the mean titer from four or five mice. (A) Viral titers on day 5 p.i. in the brains (left panel) and livers (right panel) of mice inoculated by the intracranial route with 6,000 PFU of rMHV-A59S-HE$^+$, rMHV-A59S-HE^0, and rMHV-A59S-HE$^-$. (B) Viral titers on days 3, 5, and 7 p.i. in the brains (left panel) and livers (right panel) of mice after intranasal inoculation with 10,000 PFU of rMHV-A59S-HE$^+$ (striped bars), rMHV-A59S-HE^0 (open bars), and rMHV-A59S-HE$^-$ (closed bars).

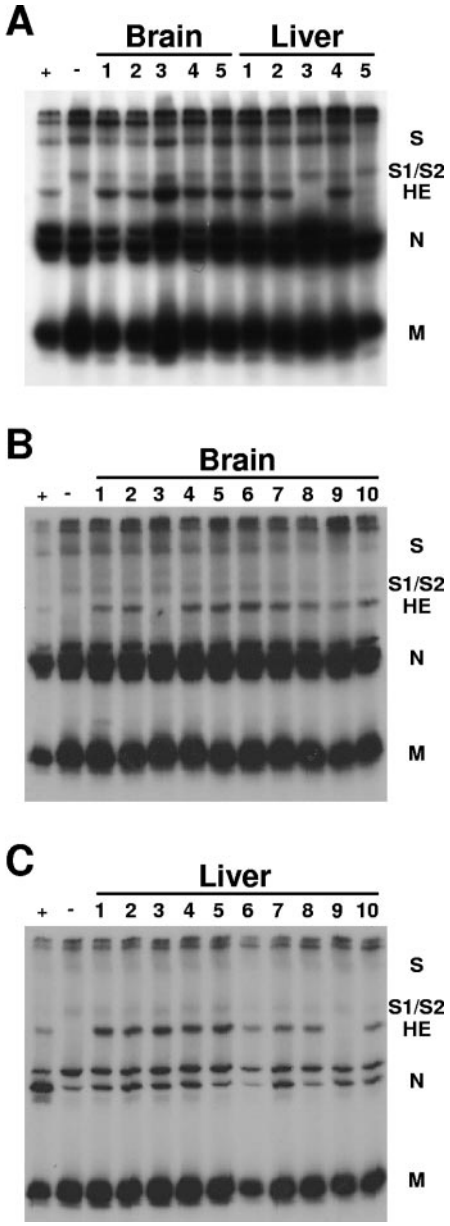

FIG. 3. Virions of rMHV-A59S-HE$^+$ and rMHV-A59S-HE^0 isolated from the livers and brains of infected mice carry HE in their envelopes. Plaque-purified viruses isolated from liver and brain lysates derived from animals infected with rMHV-A59S-HE$^+$ (A) or rMHV-A59S-HE^0 (B and C) were used to infect LR7 cells. Virus-infected cells were metabolically labeled with [^{35}S]methionine from 2 to 10 h p.i., after which time tissue culture supernatants were harvested and virus particles were collected by virus immunopurification with anti-MHV-A59 K135 serum as described in Materials and Methods and in reference 23a. Samples were analyzed in 15% sodium dodecyl sulfate-polyacrylamide gels. The first two lanes of each panel contained proteins from cells infected with rMHV-A59S-HE$^+$ (+) or rMHV-HE$^-$ (−) controls.

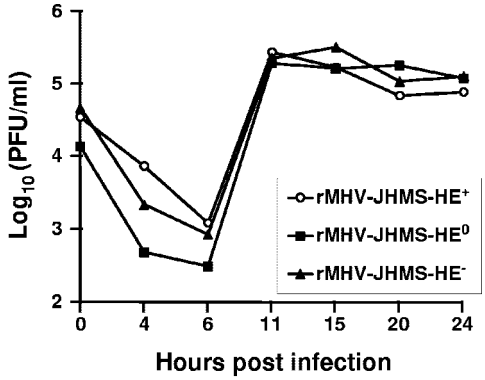

FIG. 4. Replication of rMHV-JHMS-HE$^+$, rMHV-JHMS-HE^0, and rMHV-JHMS-HE$^-$ in L2 cell cultures. L2 monolayers were infected in duplicate with the recombinant viruses at a multiplicity of infection of 1 PFU/cell. At the time points indicated, the cells were lysed and viral titers were determined. Each time point represents the mean of duplicate samples. Circles, rMHV-JHMS-HE$^+$; squares, rMHV-JHMS-HE^0; triangles, rMHV-JHMS-HE$^-$.

virulence. Theoretically, the effect of HE on viral propagation in vivo may only become overt within the context of a mouse-adapted strain and may even require the presence of other specific viral proteins. MHV-JHM, in contrast to MHV-A59, is highly neurovirulent, a property that has been attributed in large part to its S protein (32). Moreover, MHV-JHM naturally expresses the HE protein (57). These considerations prompted us to investigate the pathogenic properties of recombinant viruses that combine the expression of HE with that of the MHV-JHM S protein. Through targeted recombination, an isogenic mirror set of rMHV-A59S-HE$^+$, -HE^0, and -HE$^-$ viruses was generated, which instead of the MHV-A59 spike expressed the spike of MHV-JHM; these viruses were designated rMHV-JHMS-HE$^+$, rMHV-JHMS-HE^0, and rMHV-JHMS-HE$^-$, respectively (Fig. 1B).

One-step growth curves were created in order to determine whether these rMHVs showed any differences in replication in tissue culture that might be associated with the HE genotype. It is noteworthy that JHM replicates to significantly lower titers in tissue culture than does A59 (1) and that SJHM-RA59, an A59-based recombinant virus containing the JHM spike, displays the same distinctive in vitro growth characteristics as JHM (32). Figure 4 shows that rMHV-JHMS-HE$^+$, -JHMS-HE^0, and -JHMS-HE$^-$ replicated with similar kinetics and to similar final titers of approximately 10^5 PFU/ml as those observed previously for JHM and recombinant viruses expressing the JHM spike. Furthermore, there were no gross differences in tissue culture replication phenotypes among these viruses. As for rMHV-A59S-HE$^+$ and rMHV-A59S-HE^0 (23a), the expression of HE by the rMHV-JHMS viruses was not stable in vitro but was lost progressively during serial passaging in cell culture (not shown). Mouse inoculations were therefore performed with low-passage stocks.

Expression of HE in combination with the JHM spike protein enhances virulence. Previous studies demonstrated that the introduction of the JHM spike gene into the A59 background alone is sufficient to dramatically increase neurovirulence (32). Since the LD_{50}s of such recombinant viruses are very low to begin with, any additional potentiating effect on virulence brought about by HE expression would be difficult to assess in LD_{50} assays. Therefore, we compared the abilities of rMHV-JHMS-HE$^+$, -JHMS-HE^0, and -JHMS-HE$^-$ to cause clinical signs of disease and death following infection. Thus, C57BL/6 mice (n = 10 per virus) were injected intracranially

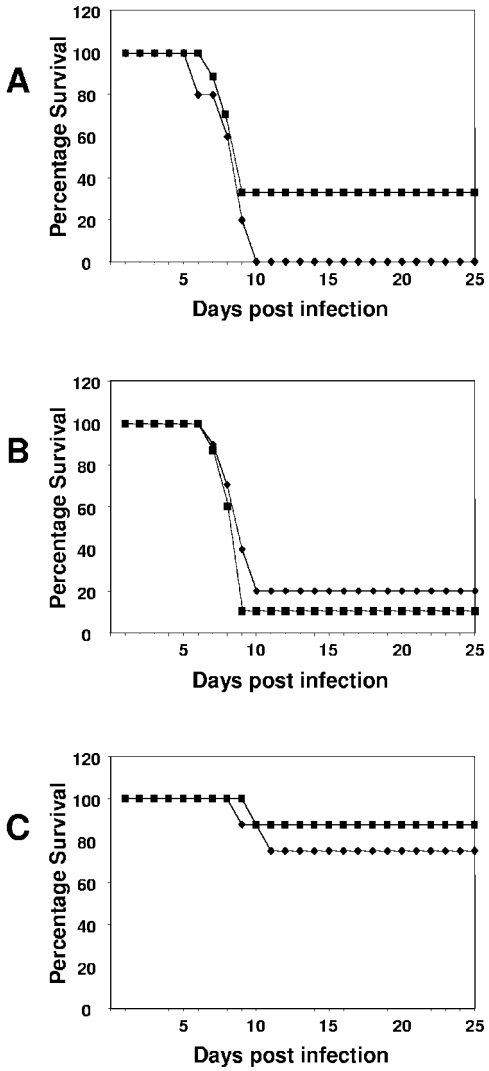

FIG. 5. Mortality of mice infected with rMHVs expressing the JHM spike. C57BL/6 mice were infected with 20 PFU/mouse of each virus and observed for 2 weeks. Percentages of survival as a function of time are shown. The data shown represent one of three separate experiments in which two independent recombinants (represented by squares and diamonds) were tested for each virus ($n = 10$ mice per virus in each experiment). (A) rMHV-JHMS-HE$^+$; (B) rMHV-JHMS-HE^0; (C) rMHV-JHMS-HE$^-$.

with 20 PFU of recombinant virus and observed daily for morbidity and lethality. The data were plotted as percentages of mice surviving as a function of time. rMHV-JHMS-HE$^+$- and -JHMS-HE^0-infected mice showed clinical signs of MHV infection, such as hunched posture, ruffled fur, and a waddling gait, beginning on day 4 or 5 p.i., while only a minority of those infected with rMHV-JHMS-HE$^-$ exhibited clinical signs, generally at later time points during the course of infection. As shown in Fig. 5, rMHV-JHMS-HE$^+$- and -JHMS-HE^0-inoculated mice succumbed to lethal infections faster, with median survival times of 8 to 9 days and survival rates between 0 and 30%. In contrast, mice infected with rMHV-JHMS-HE$^-$ displayed survival rates between 70 and 90%. The data were analyzed by the Kaplan-Meier survival method; post hoc anal-

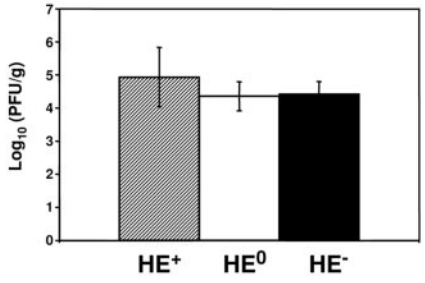

FIG. 6. Replication in C57BL/6 mice of rMHV-JHMS-HE$^+$, rMHV-JHMS-HE^0, and rMHV-JHMS-HE$^-$. Infectious viruses were titrated from brain lysates derived from mice on day 5 postinfection with 20 PFU of rMHV-JHMS-HE$^+$ (striped bar), rMHV-JHMS-HE^0 (open bar), and rMHV-JHMS-HE$^-$ (filled bar). Each bar represents the average for eight mice. The titers are expressed as $\log_{10}$(PFU/g).

ysis of variance using the Scheffe test for survival showed that the differences in survival rates for each independent recombinant of rMHV-JHMS-HE$^-$ compared to each of the other HE-expressing viruses were statistically significant ($P < 0.0001$ in each case). Perhaps most surprisingly, no differences in survival time and survival rate were observed between groups of mice infected with rMHV-JHMS-HE$^+$ or -JHMS-HE^0. These findings suggest that a structurally intact HE, whether enzymatically active or not, in combination with the MHV-JHM spike, has a significant impact on the disease outcome.

Replication and distribution of viral antigens of recombinant viruses expressing HE and the MHV-JHM spike. To further investigate the basis for the HE-mediated increase in virulence, rMHV-JHMS-HE$^+$, -JHMS-HE^0, and -JHMS-HE$^-$ were compared with each other with respect to the ability to replicate in vivo. Mice were inoculated intracranially with 20 PFU of each of the recombinant viruses and sacrificed on day 5 p.i. Brain and liver homogenates were assayed for infectious virus by plaque assays. rMHVs expressing the MHV-JHM spike are known to replicate poorly in the liver (29, 32). Thus, as anticipated and consistent with our previous observations (29, 32), the replication of all recombinant viruses was minimal in this organ. However, the three types of recombinant viruses replicated efficiently and to the same extent in the brain (Fig. 6). These data give further support to our previous observation that viral titers in the brain are not always an accurate reflection of neurovirulence (32, 50). However, major differences between HE-expressing and HE-deficient viruses became evident upon immunohistochemical analysis of sagittal brain sections of infected mice. Sections from animals infected with rMHV-JHMS-HE$^+$ and -JHMS-HE^0 displayed extensive viral antigen spread in all the major regions of the brain where MHV antigen is typically observed, including the olfactory bulb, subiculum, basal forebrain, basal ganglia, hypothalamus, midbrain, and medulla. Brain sections from rMHV-JHMS-HE$^-$-infected animals showed a similar regional distribution of viral antigen, but in striking contrast, antigen-positive cells were limited to small foci. Representative sections from the midbrains and basal forebrains of animals infected with each virus are shown in Fig. 7. Overall, our data demonstrate that HE expression in combination with the MHV-JHM spike pro-

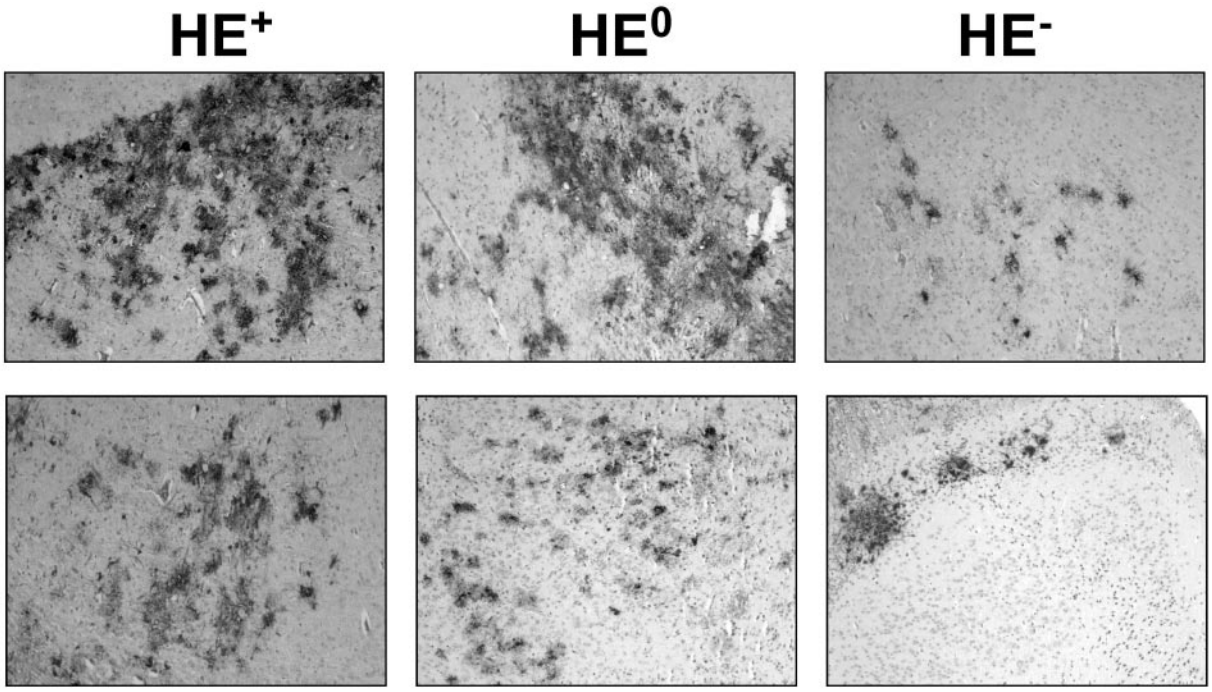

FIG. 7. Viral antigen spread in the brains of mice infected with rMHV-JHMS-HE$^+$, rMHV-JHMS-HE^0, and rMHV-JHMS-HE$^-$ on day 5 p.i. Viral antigen was detected in sagittal brain sections by staining with an antinucleocapsid monoclonal antibody as described in Materials and Methods. The top panels show sections from the midbrain, and the bottom panels show sections from the basal forebrain. From left to right, sections of animals infected with rMHV-JHMS-HE$^+$, rMHV-JHMS-HE^0, and rMHV-JHMS-HE$^-$ are shown. Magnification, ×10.

motes viral dissemination in the brain, which likely leads to enhanced neurovirulence.

Nearly all mice surviving infection with MHV-A59 develop chronic demyelination, which peaks at about 4 weeks postinfection (21–23). In order to determine whether the expression of HE also had an effect on the development of chronic demyelination, 4-week-old C57BL/6 mice were infected intracranially with 10 PFU of rMHV-JHMS-HE$^+$, -JHMS-HE^0, or -JHMS-HE$^-$. Mice surviving the acute infection were sacrificed at 30 days p.i., and their spinal cords were recovered, sectioned, and stained with Luxol fast blue to detect demyelination (23), Only 2 of 10 mice (20%) infected with rMHV-JHMS-HE$^+$ survived, 1 of which displayed demyelination. Of the 10 mice infected with rMHV-JHMS-HE^0, 3 survived (30%), 2 of which exhibited extensive demyelination and 1 of which showed smaller demyelinating lesions. In contrast, 100% (five of five) of the mice infected with rMHV-JHMS-HE$^-$ survived infection, and none showed demyelination. Thus, extensive spread of viral antigen in the acute stages of infection also seems associated with chronic demyelination later in infection. This is consistent with previous findings in our laboratory (25) indicating that efficient spread throughout the CNS is a requirement for demyelination to develop.

DISCUSSION

Recent studies with isogenic MHV viruses generated by targeted RNA recombination have demonstrated the decisive role of the S protein in determining the host range, tissue and cell preferences, and patterns of disease (29, 32, 33, 38). It is clear, however, that viral proteins other than S contribute significantly to virulence and tropism (30; Iacono et al., manuscript in preparation). One obvious candidate is HE, an accessory envelope glycoprotein with sialate-*O*-acetylesterase activity which is closely related to subunit 1 of influenza C virus HEF (24). In contrast to S, HE has attracted only limited attention so far, and its function during infection is poorly understood. The data that are available are contradictory, with some studies reporting a loss of HE expression during experimental infections of mice (60) and others describing a strong selection for HE expression in vivo (49). Yokomori et al. (58) compared the neuropathogenicities of two biologically selected JHM variants, JHM(2) and JHM(3), that differ in HE expression levels. Although their observations were interpreted to indicate that HE influences the rate of virus spread and/or tropism (58), the data are essentially inconclusive, as the viruses employed in the study were nonisogenic, and as specifically mentioned in a subsequent publication by the same group (62), may well have contained additional mutations in other parts of their genomes.

Here we have addressed the effect of HE expression during in vivo infection by using two sets of isogenic A59-based recombinant viruses, one with the autologous A59 S gene and the other carrying the S gene of JHM. The viruses within each set differ exclusively in HE expression, with HE$^+$ viruses producing a wild-type, and hence fully functional, HE protein, HE^0 viruses expressing a structurally intact but enzymatically inactive HE, and HE$^-$ viruses producing no HE at all (note that the last two viruses actually do carry the full-length gene and synthesize mRNA2b). For each type of virus, we studied two

independently isolated recombinants to ensure that spontaneous mutations in other parts of the genome did not skew the data. We found that HE, in combination with the S protein of MHV-A59, did not affect virulence or pathogenesis. The three types of recombinant viruses tested (rMHV-A59S-HE$^+$, -HE^0, and -HE$^-$) were similar to each other and to the parental wild-type strain MHV-A59 with respect to tropism, replication in the brain and liver, viral antigen spread, and histopathology. However, in combination with the S protein of MHV-JHM, HE expression markedly increased the neurovirulence of the viruses. Perhaps most surprisingly, this effect occurred irrespective of whether the HE protein produced was enzymatically active or not. In survival assays involving low-dose intracranial inoculation, the recombinant viruses rMHV-JHMS-HE$^+$ and rMHV-JHMS-HE^0 behaved similarly and displayed pathogenic properties clearly different from those of rMHV-JHMS-HE$^-$. Mice that had received rMHV-JHMS-HE$^+$ and rMHV-JHMS-HE^0 quickly succumbed to the infection, displaying very low survival rates. In contrast, of the mice inoculated with rMHV-JHMS-HE$^-$, 70 to 90% survived with minimal signs of disease at any time, and those that did develop a lethal infection displayed significantly prolonged survival times. Consistent with these findings, viruses expressing JHM S in combination with either the wild-type or enzymatically inactive HE displayed far more extensive dissemination in the brain than those expressing JHM S alone.

The finding that the effect of HE on viral spread is codependent on S is unexpected and requires further discussion. In any case, the lack of an HE-related phenotype in the rMHV-A59S-HE$^+$ and -HE^0 viruses which express MHV-A59 S cannot be explained by a loss of HE expression during in vivo replication. Almost all rMHV-A59S-HE$^+$ and -HE^0 viruses that were plaque purified from brains and livers of infected animals produced HE and incorporated the protein into their virions. Of the rMHV-A59S-HE$^+$ viruses in these tissues, the vast majority still expressed a functional *O*-acetylesterase.

Our observations might also seem at odds with the results of an earlier study by Zhang et al. (62) which aimed at in vivo complementation of MHV-A59 with HE expressed from a defective interfering RNA (A59-DE-HE). They reported a reduction of liver pathology in infected mice that had been coinoculated intracranially with A59-DE-HE compared to that in mice that had been coinoculated with a control defective interfering virus (A59-DE-CAT) or had received the standard virus only. Their findings, however, should be regarded with caution, as HE expression must have been transient. Moreover, an (early) inhibitory effect through interference with in vivo replication of the standard virus mediated by A59-DE-HE, but not by A59-DE-CAT, may well have altered the course of the disease rather than HE expression per se.

Our current data suggest that HE and S, when properly matched, cooperate during entry. Such cooperation between viral envelope proteins is reminiscent of the situation in orthomyxo- and certain paramyxoviruses. These viruses also possess two types of spike and use Sia as a receptor determinant. In influenza A and B viruses, the hemagglutinin (HA) mediates virion binding to Neu5Ac-containing cell surface receptors and membrane fusion, while the neuraminidase is a Neu5Ac-specific RDE. In paramyxoviruses, there is a different division of roles, with a hemagglutinin-neuraminidase (HN) carrying receptor-binding as well as RDE activity and with the other type of spike, F, dedicated to fusion.

It has been suggested that in group II coronaviruses, S is the major Sia-binding protein and HE mainly functions as an RDE (17, 42, 57). This view, however, is difficult to reconcile with the present observation that an enzymatically inactive HE also enhances viral spread; our findings would, in fact, be best explained by HE acting as a second receptor-binding protein after all. This would be consistent with (i) the results of others, who have demonstrated that HE proteins of various group II coronaviruses possess a Sia-subtype-specific lectin activity (42, 47); (ii) our own recent confirmation that in MHV-DVIM, HE and not S is responsible for hemagglutination (M. A. Langereis, A. L. W. van Vliet, and R. J. de Groot, unpublished data); and (iii) our observation that esterase-deficient HE-Fc chimeras of BCoV and MHV-DVIM retain Sia-specific lectin activity, as determined by hemagglutination assays and/or histochemical staining of tissue sections (M. A. Langereis and R. J. de Groot, unpublished data).

The data fit a speculative model in which virions first attach to cells via HE-mediated binding to Sia receptor determinants. This would provide S the opportunity to recruit its own specific receptor and to induce fusion and entry. The entry of MHV-A59 critically requires binding of its S protein to CAECAM1a, a CAECAM isoform abundant in the liver and colon but present only at very low levels in the murine CNS. Conceivably, in the absence of this receptor, rMHV-A59S-HE$^+$ and -HE^0 virions might be effectively concentrated at the cell surface through HE-Sia interactions, but infection would still not proceed beyond attachment. To explain the fact that HE also did not enhance the dissemination of rMHV-A59S-HE$^+$ and -HE^0 in the liver, we have to assume either that Neu4,5Ac$_2$ is not produced (or is produced only in small quantities) by the target cells or that the binding of A59 S to CAECAM1a is already sufficient to guarantee efficient attachment and/or cell-to-cell spread, thus making HE dispensable. In fact, the expression of HE in combination with A59 S seems to provide a selective disadvantage, as indicated by the fact that the proportion of rMHV-A59S-HE$^+$ viruses that had lost acetylesterase activity significantly increased from 3% in the brain, i.e., the initial site of infection, to 12.5% in the liver ($P < 0.05$).

The S protein of MHV-JHM differs significantly from that of MHV-A59 in terms of receptor selectivity and fusion activity. It is highly unstable, as demonstrated by the more rapid dissociation of S1-S2 heterodimers under physiological conditions, and as an apparent consequence, is far more fusogenic (12). Although MHV-JHM uses CAECAM1a as a receptor, at least in vitro, it primarily infects the CNS. This discrepancy may be attributed to the increased fusogenicity of its S protein, which might allow infection even under conditions of low receptor density or fusion into some cell types by receptor-independent pathways. Alternatively, the virus might enter brain cells either by using other CEACAM isotypes or by another novel receptor altogether, or even via a receptor-independent fusion mechanism. Imaginably, in all of these scenarios, attachment to the cell surface via HE could enhance the infection efficiency.

This is the first study to unambiguously demonstrate a function of HE during infection in vivo. Although in the brain the RDE activity of HE does not seem to be required, we assume that

the sialate-*O*-acetylesterase activity will make a difference during natural MHV infection. In the main target tissues, the respiratory and enteric tracts, Neu4,5Ac$_2$ receptor determinants may be more abundantly present on both cell-associated and non-cell-associated glycoconjugates, and consequently, the need for enzymatic virion detachment might be more urgent.

ACKNOWLEDGMENTS

This work was supported by NIH grant AI17418 (S.R.W.). A.L. was supported by The Netherlands Digestive Disease Foundation project WS98-41.

We thank Masha Greuter and Arno van Vliet for excellent technical help.

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