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The Spike Glycoprotein of Murine Coronavirus MHV-JHM Mediates Receptor-Independent Infection and Spread in the Central Nervous Systems of *Ceacam1a*$^{-/-}$ Mice$^{\nabla}$

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The MHV-JHM strain of the murine coronavirus mouse hepatitis virus is much more neurovirulent than the MHV-A59 strain, although both strains use murine CEACAM1a (mCEACAM1a) as the receptor to infect murine cells. We previously showed that *Ceacam1a*$^{-/-}$ mice are completely resistant to MHV-A59 infection (E. Hemmila et al., J. Virol. 78:10156–10165, 2004). In vitro, MHV-JHM, but not MHV-A59, can spread from infected murine cells to cells that lack mCEACAM1a, a phenomenon called receptor-independent spread. To determine whether MHV-JHM could infect and spread in the brain independent of mCEACAM1a, we inoculated *Ceacam1a*$^{-/-}$ mice. Although *Ceacam1a*$^{-/-}$ mice were completely resistant to i.c. inoculation with 10^6 PFU of recombinant wild-type MHV-A59 (RA59) virus, these mice were killed by recombinant MHV-JHM (RJHM) and a chimeric virus containing the spike of MHV-JHM in the MHV-A59 genome (SJHM/RA59). Immunohistochemistry showed that RJHM and SJHM/RA59 infected all neural cell types and induced severe microgliosis in both *Ceacam1a*$^{-/-}$ and wild-type mice. For RJHM, the 50% lethal dose (LD_{50}) is $<10^{1.3}$ in wild-type mice and $10^{3.1}$ in *Ceacam1a*$^{-/-}$ mice. For SJHM/RA59, the LD_{50} is $<10^{1.3}$ in wild-type mice and $10^{3.6}$ in *Ceacam1a*$^{-/-}$ mice. This study shows that infection and spread of MHV-JHM in the brain are dependent upon the viral spike glycoprotein. RJHM can initiate infection in the brains of *Ceacam1a*$^{-/-}$ mice, but expression of mCEACAM1a increases susceptibility to infection. The spread of infection in the brain is mCEACAM1a independent. Thus, the ability of the MHV-JHM spike to mediate mCEACAM1a-independent spread in the brain is likely an important factor in the severe neurovirulence of MHV-JHM in wild-type mice.

Mouse hepatitis virus (MHV), an enveloped virus with a positive-sense RNA genome in the *Coronaviridae* family, has been studied widely as a model of viral pathogenesis. Strains of MHV differ in tissue tropism and virulence in mice. While MHV-A59 is both hepatotropic and neurotropic, MHV-JHM is primarily neurotropic and is much more neurovirulent than MHV-A59 (4, 22). When inoculated intracerebrally (i.c.), MHV-A59 and MHV-JHM both replicate in the central nervous systems (CNS) of susceptible mice, yet these viruses differ markedly in pathogenesis (18, 34, 38). MHV-A59 causes hepatitis, mild encephalitis, and subacute demyelination, whereas MHV-JHM causes acute, fatal encephalitis and, in mice that survive acute infection, chronic demyelination. The spike glycoprotein of MHV-JHM is a major determinant of neurovirulence, although other viral genes also contribute to neurovirulence (18, 34, 35, 38). The spike glycoprotein of coronaviruses is responsible for viral attachment to the cellular receptor and fusion of the viral and cellular membranes, resulting in virus entry. In addition, the spike glycoprotein is expressed on the surface of infected cells, where it mediates cell-to-cell fusion, resulting in spread of virus by syncytium formation.

MHV-A59 and MHV-JHM virions can infect only murine cells, and they use murine CEACAM1a (mCEACAM1a) glycoproteins, in the immunoglobulin protein superfamily, as their receptors to enter susceptible cells (6–8). The *Ceacam1a* gene is expressed as four major isoforms with either two or four immunoglobulin-like exodomains and a long or short cytoplasmic tail, and all four isoforms are functional receptors for MHV strains (7, 49). A monoclonal antibody (MAb) to the N-terminal domain (D1) of mCEACAM1a (MAb-CC1) recognizes only mCEACAM1a proteins (9, 46). Pretreatment of murine cells with MAb-CC1 prior to inoculation with MHV-A59, MHV-JHM, and other strains blocks virus attachment and infection (8, 16, 27, 29, 46). Treatment of cells with MAb-CC1 after inoculation with MHV-A59 also blocks cell-to-cell spread of infection. In contrast, cell-to-cell spread of MHV-JHM infection in murine cells is not blocked by MAb-CC1, although virion entry is blocked (26, 27). Similarly, MHV-JHM infection can spread from infected murine cells (DBT or 17Cl-1) to hamster (BHK) or rabbit (RK13) cells that do not express mCEACAM1a, even though MHV-JHM cannot infect these cells (13, 27, 30, 43). This so-called "receptor-independent spread" (RIS) of MHV-JHM has been demonstrated using neural and nonneural cell lines and primary neural cells (13, 20, 26, 27, 30, 43). In contrast, the less neurovirulent strain

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MHV-A59 produces minimal syncytia by RIS in vitro (13, 43). MAbs to the spike of MHV-JHM block RIS, and recombinantly expressed MHV-JHM spike mediates receptor-independent cell-to-cell fusion (13, 27, 42, 43). The mCEACAM1a-independent fusion activity of spike correlates with reduced stability of the interaction between the receptor-binding domain (S1) and the fusion domain (S2) and with neurovirulence (10, 20, 30). Thus, in vitro, mCEACAM1a is required for initial cell entry by both MHV-A59 and MHV-JHM and for syncytium formation by MHV-A59 but not MHV-JHM.

Treatment of newborn BALB/c mice with MAb-CC1 before and after i.c. inoculation with MHV-A59 prevents virus replication in the nose, brain, and liver (39). Because receptor blockade by MAb-CC1 is rarely complete, especially in vivo, further studies using *Ceacam1a* gene-disrupted mice were undertaken to evaluate the importance of mCEACAM1a during in vivo infection with MHV. The first targeted disruption of the *Ceacam1a* gene in BALB/c mice (called p/p) resulted in markedly reduced expression of the four-domain isoform and altered ratios of the different isoforms of mCEACAM1a in different tissues (3). When inoculated intranasally (i.n.) with a high dose (10^8 PFU) of MHV-A59, 8- to 12-week-old homozygous p/p mice did not develop clinical signs and had fewer and smaller lesions and lower virus yield in the liver than wild-type BALB/c mice (3). This dose of MHV-A59 is lethal for wild-type BALB/c mice. Subsequently, we developed mice in which expression of the *Ceacam1a* gene was ablated completely (17, 24). Three-week-old homozygous (*Ceacam1a*$^{-/-}$) mice on a C57BL/6 background were completely resistant to infection with 10^6 PFU of MHV-A59 by both the i.n. and i.c. routes (17). Thus, mCEACAM1a is critical for MHV-A59 infection of C57BL/6 mice.

MHV-JHM infects primarily the CNS of susceptible mice, where mCEACAM1a expression is much lower than in other tissues (15, 16, 29, 45). Although by immunohistochemistry mCEACAM1a could be detected only in the brain on the luminal surface of endothelial cells, MHV-JHM infects neurons, astrocytes, microglia, ependymal cells, and oligodendrocytes (15, 23, 31, 33, 41). mCEACAM1a mRNA can be detected in whole-brain lysates and at very low levels in neuronal cultures (unpublished observations). Freshly isolated microglia and cultured microglia and oligodendrocytes express mCEAC AM1a and are susceptible to infection with MHV-A59 or MHV-JHM (16, 26, 36). Infection of these cells with MHV-A59 or MHV-JHM can be blocked with MAb-CC1; however, spread of MHV-JHM by syncytium formation is not blocked by MAb-CC1 in primary neural cultures (16, 26, 36). Previous studies have addressed the importance of RIS in the neurovirulence of MHV-JHM by using in vitro models of neural cells. However, explanted neural cells differ from cells in the brain, particularly in the expression of mCEACAM1a. Therefore, we used *Ceacam1a*$^{-/-}$ mice to evaluate the potential for MHV-JHM to infect and spread in the CNS in vivo, independent of mCEACAM1a.

MATERIALS AND METHODS

Viruses. The recombinant viruses used in this study, including recombinant wild-type MHV-A59 (RA59) (S_{A59}R13, wtR13), recombinant MHV-JHM (RJHM), and a chimeric virus containing the spike of MHV-JHM in the MHV-A59 genome (SJHM/RA59) (S_4R22), were constructed by targeted RNA recombination, as previously described (28, 34, 35). Viruses were propagated in 17Cl-1 cells and titrated by plaque assay in L2 or 17Cl-1 cells (12, 17).

Susceptibility of wild-type and *Ceacam1a*$^{-/-}$ C57BL/6 mice to MHV infection. Wild-type C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) and homozygous mice with complete disruption of the *Ceacam1a* gene on a C57BL/6 background were used in this study. The generation of *Ceacam1a*$^{-/-}$ mice and their resistance to infection by MHV-A59 after three backcrosses on the C57BL/6 background were described previously (17, 24). The *Ceacam1a*$^{-/-}$ mice used in this study had been backcrossed on the C57BL/6 background 10 additional generations since their initial characterization (17). *Ceacam1a*$^{-/-}$ mice were housed and bred in the Center for Comparative Medicine at the University of Colorado Health Sciences Center using protocols approved by the Institutional Animal Care and Use Committee. Mice were genotyped as previously described to confirm complete disruption of the *Ceacam1a* gene (17).

Four-week-old wild-type C57BL/6 or *Ceacam1a*$^{-/-}$ mice were anesthetized intraperitoneally with tribromoethanol (Sigma-Aldrich, Milwaukee, WI), and 25 μl of virus diluted in phosphate-buffered saline containing 0.75% bovine serum albumin (dilution buffer) was inoculated i.c. in the right cerebral hemisphere by using a 30-gauge needle and a 1-ml syringe in a Tridak stepper repetitive pipette (Indicon, Inc., Brookfield Center, CT). Inoculated mice housed in individually vented biocontainment cages were fed standard rodent chow and water ad libitum, and apple pieces were placed on the cage floor to prevent dehydration. Wild-type C57BL/6 and *Ceacam1a*$^{-/-}$ mice were inoculated i.c. with dilution buffer alone or various doses of RA59, RJHM, or SJHM/RA59.

Five wild-type mice per dose were euthanized with an overdose of inhaled isofluorane or tribromoethanol delivered intraperitoneally followed by exsanguination on day 5 after inoculation, which was previously reported to be the peak of viral replication (34). *Ceacam1a*$^{-/-}$ mice were euthanized on days 5, 7, 8, and 9 after inoculation. Brain and liver were harvested for quantification of infectious virus, detection of viral antigen, and histopathological analysis. To determine the 50% lethal dose (LD_{50}) for each virus in wild-type and *Ceacam1a*$^{-/-}$ mice, 5 to 10 mice per virus dose were monitored for 30 days for signs of neurological disease, including weight loss, lethargy, ruffled fur, hunched posture, an elevated tail, spasticity, and hind limb paresis. Mice were euthanized if they lost more than 15% of their body weight or appeared moribund. The LD_{50} ($\log_{10}$ PFU) was calculated by the Reed-Muench method (37).

For i.n. inoculation, 4-week-old C57BL/6 and *Ceacam1a*$^{-/-}$ mice were anesthetized with an intraperitoneal injection of tribromoethanol and inoculated i.n. with 10 μl of RJHM (10,000 PFU) or with dilution buffer alone. Mice were monitored until the liquid had all been inhaled. Inoculated mice were housed and monitored as described above.

Quantification of viral yield in mouse brain tissue. Mice were anesthetized deeply with tribromoethanol or isofluorane and exsanguinated by cardiac puncture. The left hemisphere of each brain was used for histopathology and immunohistochemistry and the right for virus titration. For virus titration, the right hemisphere was weighed, rinsed in phosphate-buffered saline, flash frozen in Dulbecco's modified Eagle medium containing 10% fetal bovine serum and antibiotics, and stored at −80°C. The tissue was later thawed, minced, and frozen and thawed again. Debris was removed by centrifugation at 10,000 × *g* for 10 min prior to titration of infectious virus in the supernatant medium by plaque assay on 17Cl-1 cells (12, 17). Virus yield was reported as PFU/g of brain tissue.

Histopathology. Routinely processed paraffin-embedded sections of brain, stained with hematoxylin and eosin, were examined by one of us (H.B.-O.) without prior knowledge of the group assignment, and a severity score was assigned to the brain lesions as follows: 0, no apparent changes; 1, minimal leukocyte infiltration in meninges and/or cerebrum; 2, mild meningoencephalitis (ME); 3, moderate ME; 4, severe ME; 5, very severe ME with malacia.

Immunohistochemistry. For immunohistochemistry, the left hemisphere of the brain and a single lobe of liver were fixed in 10% phosphate-buffered formalin and embedded in paraffin. Five- to six-micrometer-thick paraffin sections were deparaffinized using Histoclear (National Diagnostics, Atlanta, GA), rehydrated, and subjected to antigen retrieval by (i) incubation in target retrieval solution, pH 9.0 (DAKO, Carpentaria, CA), (ii) incubation in citrate buffer, pH 6.0 (ScyTek Laboratories, Logan, UT), for 25 min at 90°C, followed by a 20-min cooling period at room temperature, or (iii) treatment with proteinase K (DAKO) for 15 min at room temperature, with the method of choice depending on the primary and secondary antibody combination employed. A MAb specific for the nucleocapsid protein of MHV (MAb clone 1-16-1) was kindly provided by J. Leibowitz (Texas A&M University, College Station, TX). This MAb gave equally strong signals with all MHV strains and all three antigen retrieval methods. Dual immunolabeling was performed as previously described (1, 2). The slides were incubated with the mouse MAb 1-16-1 and one of each of the following four rabbit antibodies that recognize cell-specific markers: OLIG2

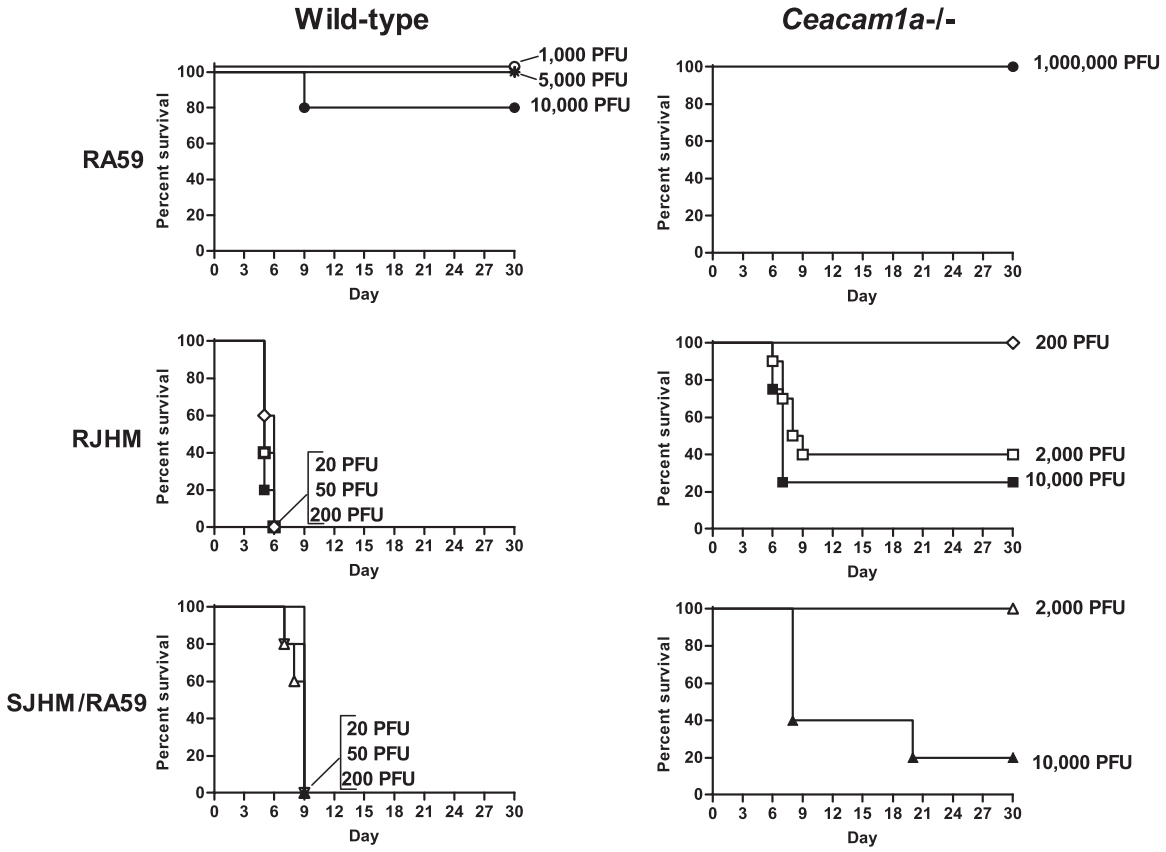


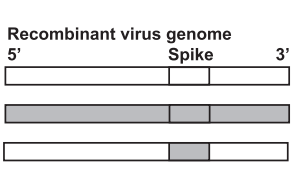
FIG. 1. Survival of wild-type and *Ceacam1a*$^{-/-}$ mice following i.c. inoculation with recombinant MHV. Five wild-type C57BL/6 and 5 to 10 *Ceacam1a*$^{-/-}$ mice per dose were inoculated i.c. with with RA59, RJHM, or SJHM/RA59 at the doses indicated. Mice were monitored for 30 days for weight loss and clinical signs and euthanized if moribund. Mice that were inoculated with dilution buffer alone had 100% survival (data not shown).

(Chemicon, Inc., Temecula, CA) for oligodendrocytes, GFAP (DAKO) for astrocytes, neurofilament heavy chain (Abcam, Cambridge, MA) for neurons, or Iba1 (WAKO Chemicals, Inc., Richmond, VA) for microglia. This was followed by each of the following: (i) biotin-conjugated goat anti-rabbit immunoglobulin G (Vector Labs), (ii) streptavidin-alkaline phosphatase conjugate (Vector Labs), and (iii) mouse Envision-horseradish peroxidase reagent (DAKO). MAb 1-16-1 was visualized with 3-amino-9-ethylcarbazole (red product; DAKO) while the four cell-specific antibodies were visualized with BCIP-NBT (5-bromo-4-chloro-3-indolylphosphate–nitroblue tetrazolium) substrate (dark-magenta product; DAKO). The sections were lightly counterstained with Mayer's hematoxylin (Scytek Laboratories), mounted with coverslips using Glycergel (DAKO), sealed, and examined with an Olympus BX41 light microscope (Olympus, Center Valley, PA). Photomicrographs were acquired with an Olympus Q-Color 3 camera and associated computer software (Olympus). The numbers of MHV antigen-positive cells and the regions of the brains that were infected were scored, by a person (H.B.-O.) blinded to the animal and group number, on a scale of 0 to 6 as follows: 0, no apparent positive cells; R, rare, one or two positive cells in the entire section; 1, small numbers of positive cells in one or a few scattered areas or dispersed; 2, small numbers of positive cells in three or more regions of the brain; 3, moderate numbers of positive cells in two or three restricted regions of the brain; 4, large numbers of positive cells in two or three regions; 5, large numbers of positive cells in 50 to 75% of the brain section; 6, very large numbers of intensely positive cells in all regions of the brain. For sections of liver, a severity score was assigned on a scale of 0 to 6 based on the number of cells that were positive for viral antigen throughout the section.

RESULTS

Effects of i.c. inoculation of recombinant MHVs in wild-type C57BL/6 and *Ceacam1a*$^{-/-}$ mice. Four-week-old wild-type C57BL/6 or *Ceacam1a*$^{-/-}$ mice were inoculated i.c. with 25 μl of dilution buffer alone or various doses of RA59, RJHM, or SJHM/RA59. RA59 and RJHM are recombinant viruses containing the genomes of MHV-A59 and MHV-JHM, respectively, and SJHM/RA59 is a chimeric virus with the spike of MHV-JHM in the RA59 genome. Mice were monitored for death or signs of neurological disease, including weight loss, lethargy, ruffled fur, hunched posture, an elevated tail, or hind limb paresis. Wild-type mice survived after i.c. inoculation with dilution buffer alone or with 1,000 PFU or 5,000 PFU of RA59 (Fig. 1 and data not shown). Eighty percent of the wild-type mice that were inoculated with 10,000 PFU of RA59 also survived. In contrast, no wild-type mice survived after i.c. inoculation with 20 PFU, 50 PFU, or 200 PFU of RJHM or SJHM/RA59. Inoculation of wild-type mice with RJHM at these three doses resulted in death of all of the mice on days 5 and 6 after inoculation. In contrast, inoculation of wild-type mice with SJHM/RA59 with the same doses resulted in death of the mice on days 7, 8, and 9 after inoculation. Thus, wild-type mice inoculated with RJHM died 1 to 4 days earlier than those inoculated with SJHM/RA59 (Fig. 1). These observations confirm previous studies of 4-week-old C57BL/6 mice with RA59, RJHM, and SJHM/RA59 (18, 34).

All *Ceacam1a*$^{-/-}$ mice inoculated with dilution buffer alone survived (data not shown), and as expected, *Ceacam1a*$^{-/-}$



Virus	$Log_{10}LD_{50}$ (day to death) Wild-type	*Ceacam1a-/-*
RA59	>4.0	>6.0
RJHM	<1.3 (5-6)	3.1 (6-9)
SJHM/RA59	<1.3 (7-9)	3.6 (8)

FIG. 2. Virulence of recombinantly derived MHV strains in wild-type C57BL/6 and *Ceacam1a*$^{-/-}$ mice. The genomes of the parental RA59 and RJHM strains and the chimeric virus SJHM/RA59 are diagrammed on the left. Open bar, MHV-A59 genes; filled bar, MHV-JHM genes. The $\log_{10}$ LD_{50} and days to death after inoculation of C57BL/6 or *Ceacam1a*$^{-/-}$ mice are shown for each virus.

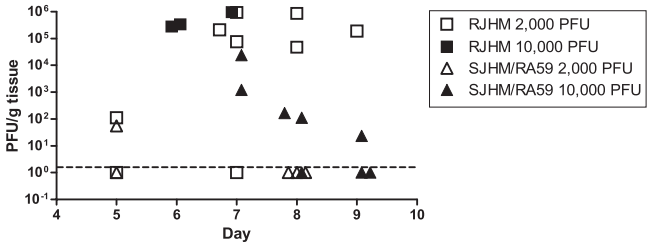


FIG. 3. Virus yields from brains of *Ceacam1a*$^{-/-}$ mice. Mice were inoculated i.c. with 2,000 PFU or 10,000 PFU of RJHM or SJHM/RA59 viruses. Virus in brain homogenates was titrated by plaque assay and was reported as PFU/g of brain tissue. The dashed line indicates the limit of detection of the plaque assay.

mice were completely resistant to i.c. inoculation with 10^6 PFU of RA59 (Fig. 1) (17). In contrast, i.c. inoculation of *Ceacam1a*$^{-/-}$ mice with 2,000 PFU or 10,000 PFU of RJHM was lethal for 70% or 80% of the mice, respectively (Fig. 1). The *Ceacam1a*$^{-/-}$ mice were susceptible to death with 10,000 PFU, but not 2,000 PFU, of SJHM/RA59 (Fig. 1). Thus, the spike glycoprotein of MHV-JHM was required for mCEACAM1a-independent infection in vivo. Although the signs of neurological disease in the RJHM- and SJHM/RA59-infected *Ceacam1a*$^{-/-}$ mice were the same as those in wild-type C57BL/6 mice, lethal infection of *Ceacam1a*$^{-/-}$ mice required a much higher dose of RJHM or SJHM/RA59 than lethal infection of wild-type mice. In C57BL/6 mice, the LD_{50}s of RJHM and SJHM/RA59 were both <1.3 $\log_{10}$ PFU, versus 3.1 $\log_{10}$ PFU and 3.6 $\log_{10}$ PFU, respectively, in *Ceacam1a*$^{-/-}$ mice (Fig. 2).

To determine whether RJHM virions alone, without debris from infected cells, were capable of initiating infection of *Ceacam1a*$^{-/-}$ mice, we inoculated six *Ceacam1a*$^{-/-}$ mice with 5,000 PFU of RJHM that was filtered through a 0.2-μm filter. Two of the mice died on days 6 and 9, and a third mouse had mild clinical signs and failed to gain weight through day 20 but recovered with a strong antibody response measured on day 30 (data not shown). Thus, MHV-JHM virions alone, without cells or cellular debris, were sufficient for infection of the brains of *Ceacam1a*$^{-/-}$ mice.

Yields of RJHM and SJHM/RA59 viruses in the brains of *Ceacam1a*$^{-/-}$ mice. The titers of virus in the brains of i.c. inoculated *Ceacam1a*$^{-/-}$ mice were determined by plaque assay (Fig. 3). Very little RJHM or SJHM/RA59 was detected in the brain on day 5 after inoculation, which is the peak of virus replication in the brains of wild-type C57BL/6 mice (34). Viral yields of 5×10^4 to 9.5×10^5 PFU/g were detected in the brains of *Ceacam1a*$^{-/-}$ mice 6 to 9 days after i.c. inoculation with 2,000 PFU or 10,000 PFU of RJHM. Notably lower titers (1×10^3 to 2.3×10^4 PFU/g) of SJHM/RA59 were detected on days 7 to 9 after i.c. inoculation with 10,000 PFU. On day 5, one *Ceacam1a*$^{-/-}$ animal that had been inoculated with 2,000 PFU of SJHM/RA59 had detectable virus in the brain. However, on day 5 or 8 no virus was detected in the brains of the other *Ceacam1a*$^{-/-}$ mice that had been inoculated with 2,000 PFU of SJHM/RA59. The yield of SJHM/RA59 in the liver of *Ceacam1a*$^{-/-}$ mice on day 8 after inoculation with 10,000 PFU was below the limit of detection of our plaque assay (50 PFU/g) (data not shown).

Virus infection and spread in different cell types in brains of *Ceacam1a*$^{-/-}$ mice. Sections of brains from wild-type and *Ceacam1a*$^{-/-}$ mice taken at various time points after inoculation with RA59, RJHM, or SJHM/RA59 were immunolabeled to detect infected cells that express viral nucleocapsid protein and scored for the number of virus-infected cells and for the regions of the brains that were infected. Adjacent sections were stained with hematoxylin and eosin and assigned a histopathology score as described in Materials and Methods. Liver tissue was harvested from animals inoculated with RA59 or SJHM/RA59 and evaluated for the presence of infected cells by immunohistochemistry. As expected, wild-type mice inoculated i.c. with 10,000 PFU of RA59 had infected cells in the liver and brain (data not shown). In contrast, *Ceacam1a*$^{-/-}$ mice inoculated i.c. with 10^6 PFU of RA59 had no infected cells in the brain or liver (Table 1). The histopathology scores in the brain sections correlated well with the viral antigen scores (Table 1). There was no difference in the distributions of infected cells in the brains of wild-type C57BL/6 and *Ceacam1a*$^{-/-}$ mice after infection with the same virus, although a higher dose was required for lethal infection of the *Ceacam1a*$^{-/-}$ mice (data not shown). Two of the four mice inoculated with 10,000 PFU of SJHM/RA59 that had infected cells in the brain had very few infected cells in the liver on days 7 and 8 (Table 1). *Ceacam1a*$^{-/-}$ mice inoculated with RJHM had more severe histopathology and more infected cells than *Ceacam1a*$^{-/-}$ mice inoculated with SJHM/RA59 (Table 1). These data demonstrated that viral antigen in brain sections of *Ceacam1a*$^{-/-}$ mice infected with RJHM was much more widespread than in animals infected with SJHM/RA59. This is in agreement with previous studies of wild-type C57BL/6 mice (18).

In order to identify the specific cell types that were infected in the brains of wild-type and *Ceacam1a*$^{-/-}$ mice, brain sections were dually immunolabeled for viral nucleocapsid antigen and markers specific for neurons, microglia, astrocytes, or oligodendrocytes. There was no difference in the relative proportions of neural cell types that were infected by RA59, RJHM, and SJHM/RA59 in wild-type mice versus RJHM and SJHM/RA59 in *Ceacam1a*$^{-/-}$ mice. Therefore, we chose to show immunolabeling of viral nucleocapsid protein and cell type-specific markers from one representative *Ceacam1a*$^{-/-}$ mouse inoculated with 2,000 PFU of RJHM and harvested on day 7 (Fig. 4). In all of the animals that had positive labeling of viral antigen, over 90% of the infected cells in the brain were neurons, as judged by cell size, morphology, and staining with antibody to neurofilament heavy chain (Fig. 4A). Almost 10% of viral antigen-positive cells were identified as microglia or

TABLE 1. Histopathology and immunocytochemistry in brains of *Ceacam1a*$^{-/-}$ mice

Virus and dose (PFU)[a]	dpi[b]	Clinical signs	Histopathology score[c] in brain	Viral antigen score[c] in:	
				Brain	Liver
RJHM					
2,000	5	No	0	0	0
	7	Yes	5	6	0
	8	Yes	5	6	0
	9	Yes	3	4	NA[d]
10,000	6	Yes	5	5	NA
	6	Yes	4	5	NA
	7	Yes	5	5	NA
SJHM/RA59					
2,000	5	No	0	0	0
	8	No	0	0	0
10,000	7	Yes	4	2	1
	8	Yes	3	1	0
	8	Yes	3	2	2
	9	No	1	0	0
	20	Yes	2	R	NA
RA59					
10^6	5	No	0	0	0

[a] *Ceacam1a*$^{-/-}$ mice were inoculated i.c. with the indicated virus and dose. Results from individual mice are shown.
[b] dpi, day postinoculation.
[c] The histopathology scores and viral antigen scores are described in Materials and Methods.
[d] NA, not available.

astrocytes based on immunolabeling of Iba1 and GFAP, respectively (Fig. 4B and C). Rare oligodendrocytes had positive staining for viral antigen (Fig. 4D). Animals with widespread viral infection had notably decreased numbers of OLIG2-positive oligodendrocytes in the brain (data not shown).

Immunolabeling of Iba1, a marker for microglia, demonstrated a distinct increase in the number and size of microglia in the brains of infected mice (Fig. 5). Microgliosis was observed to occur in all infected animals, including C57BL/6 mice infected with RA59, RJHM, or SJHM/RA59 and *Ceacam1a*$^{-/-}$ mice infected with RJHM or SJHM/RA59. In contrast, microgliosis was not seen in mice that were resistant to infection, for example, *Ceacam1a*$^{-/-}$ mice that were inoculated i.c. with 10^6 PFU of RA59 (Fig. 5), or in mice inoculated with dilution buffer alone (data not shown).

Effects of i.n. inoculation of RJHM in wild-type C57BL/6 and *Ceacam1a*$^{-/-}$ mice. Three *Ceacam1a*$^{-/-}$ mice were inoculated i.n. with dilution buffer and five C57BL/6 and eight *Ceacam1a*$^{-/-}$ mice were inoculated i.n. with 10,000 PFU of RJHM. The mice were observed for 30 days for signs of neurological disease. All of the wild-type C57BL/6 mice inoculated i.n. with RJHM died by day 7 (Fig. 6). In contrast, 100% of the *Ceacam1a*$^{-/-}$ mice survived longer than 30 days after i.n. inoculation with 10,000 PFU of RJHM. All of the *Ceacam1a*$^{-/-}$ mice that were inoculated with dilution buffer alone survived (data not shown). Thus, 10,000 PFU of RJHM, which is lethal by i.c. inoculation, is not lethal by the i.n. route in *Ceacam1a*$^{-/-}$ mice.

DISCUSSION

The interaction of coronaviral envelope glycoproteins with their cellular receptors is a critical early event in virus infection. All strains of MHV tested use mCEACAM1a as a receptor to enter cells in vitro. Although MHV-JHM virions require mCEACAM1a for entry in vitro, this virus infects primarily cells in the brain, where the level of mCEACAM1a expression is very low (15, 16, 45). In this study, we show that RJHM virions, but not RA59 virions, were able to infect *Ceacam1a*$^{-/-}$ mice by the i.c. route (Fig. 1 and 2). However, at least 100-fold more RJHM virus was required for lethal infection of *Ceacam1a*$^{-/-}$ mice than wild-type mice. These data show that expression of mCEACAM1a in the brains of wild-type C57BL/6 mice promotes MHV-JHM infection but that mCEACAM1a is not absolutely required for MHV-JHM infection, since *Ceacam1a*$^{-/-}$ mice can be infected by i.c. inoculation with an increased dose of virus.

Studies using chimeric viruses showed that the spike of MHV-JHM is a major determinant of neurotropism and neurovirulence. This is substantiated by increased viral spread and immune-mediated pathology in the brains of mice infected with RJHM or SJHM/RA59 versus RA59 (18, 34, 35, 38). Using an isogenic virus with the spike of MHV-JHM in the MHV-A59 genome (SJHM/RA59), we showed that the spike of MHV-JHM is required for infection of *Ceacam1a*$^{-/-}$ mice (Fig. 1). The LD_{50} of SJHM/RA59 was threefold higher than that of RJHM in *Ceacam1a*$^{-/-}$ mice (Fig. 2). Furthermore, viral antigen was more widespread in the brains of *Ceacam1a*$^{-/-}$ mice that were inoculated with RJHM than in those inoculated with SJHM/RA59 (Table 1). These data support previous studies of C57BL/6 mice that showed that viral genes in addition to spike contribute to viral spread and immunopathology in the brain (18, 38).

These results raise two important questions. (i) How does RJHM initiate infection in murine brains in the absence of mCEACAM1a? (ii) What properties of the spike of MHV-JHM, but not MHV-A59, account for RIS of infection in *Ceacam1a*$^{-/-}$ mice? Previous studies using mCEACAM1a blocking antibody (MAb-CC1) or cells of different species demonstrated that MHV-JHM can spread by syncytium formation in the absence of mCEACAM1a (13, 20, 26, 27, 30, 43). However, in vitro, MHV-JHM virions require mCEACAM1a to enter cells (13, 26, 27). We showed that filtered RJHM virions free of cell debris and membranes can establish infection of *Ceacam1a*$^{-/-}$ mice. The reason for this discrepancy between the requirements for in vitro and in vivo receptor-independent infection is unclear. Gallagher et al. inoculated mCEACAM1a-negative hamster and rabbit cells with MHV-JHM at a multiplicity of infection of 0.2 PFU per cell and were unable to detect viral antigen after 22 h (13). In contrast, we showed that i.c. inoculation with 2,000 PFU of RJHM was required for lethal infection of *Ceacam1a*$^{-/-}$ mice. Perhaps mCEACAM1a-negative cells in vitro could be infected by a high dose of MHV-JHM. Alternatively, there might be a different receptor for MHV-JHM in the brains of *Ceacam1a*$^{-/-}$ mice that is not present on the hamster or rabbit cell lines used in the in vitro studies.

We demonstrated that RJHM and SJHM/RA59, but not RA59, are capable of infecting *Ceacam1a*$^{-/-}$ mice. Krueger et

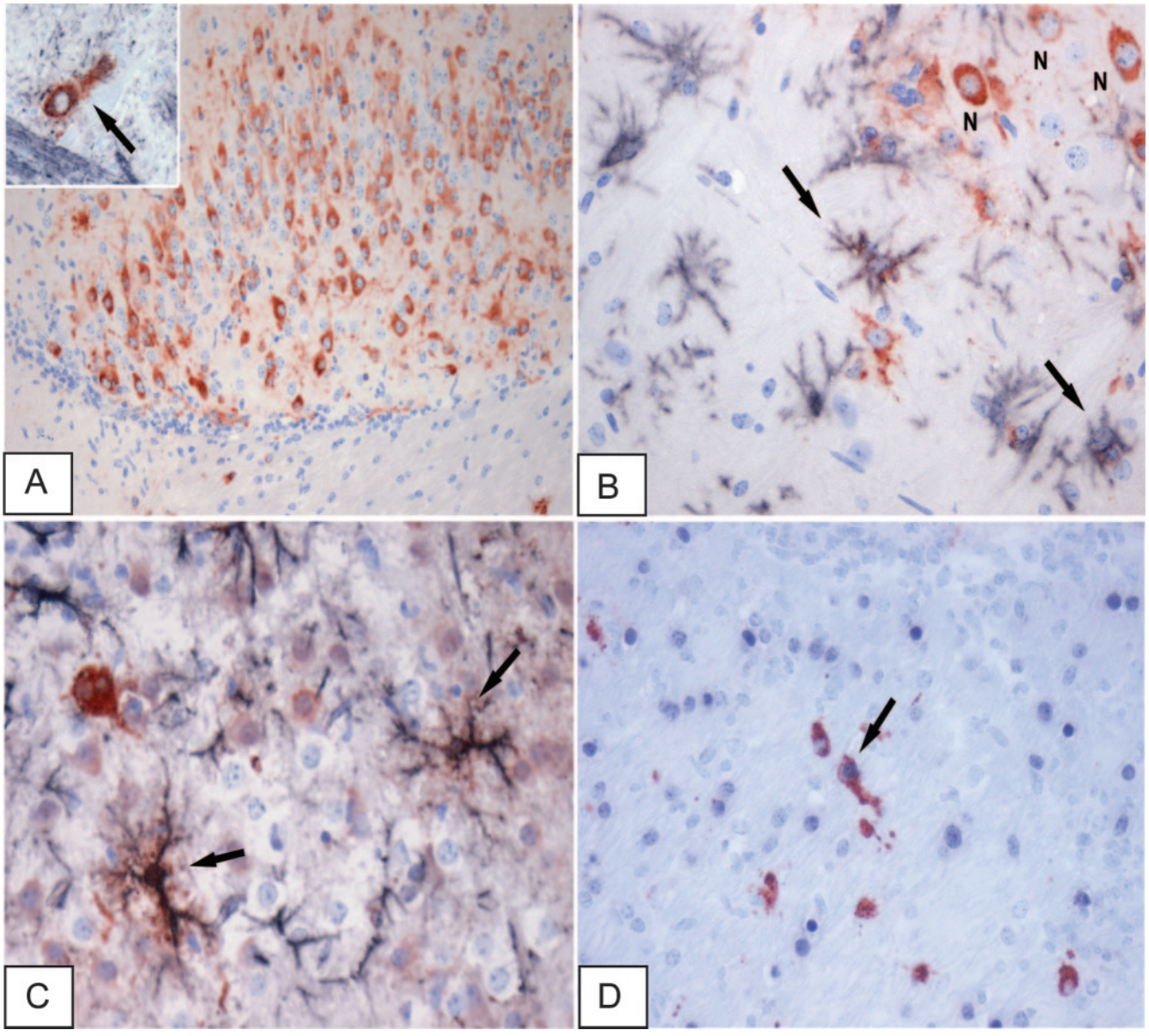


FIG. 4. MHV infection in different cell types in the brains of *Ceacam1a*$^{-/-}$ mice. Wild-type C57BL/6 and *Ceacam1a*$^{-/-}$ mice inoculated i.c. with various doses of RA59, RJHM, or SJHM/RA59 viruses were euthanized, and brains were harvested at various times after inoculation. Brain sections were analyzed by dual immunolabeling of cell-type markers (dark blue/black) and viral antigen (red). Shown here are representative photos of brain sections from a *Ceacam1a*$^{-/-}$ mouse that was inoculated i.c. with 2,000 PFU of RJHM and euthanized on day 7 after inoculation. Viral antigen was detected in (A) neurons, (B) Iba1-positive microglia, (C) GFAP-positive astrocytes, and (D) OLIG2-positive nuclear staining of oligodendrocytes. The photo in panel A consists primarily of infected neurons, as judged by cell size and morphology, with an inset of an infected neuron that is labeled with antibody to neurofilament heavy chain (dark blue/black) and viral antigen (red). Arrows indicate cells that are positive for the cell type-specific marker and viral nucleocapsid protein. Infected neurons in sections stained for the other cell type-specific markers (B, C, and D) are indicated with an "N." Actual magnifications: (A) ×125, (inset) ×350, (B, C, and D) ×350.

al. showed that the S1/S2 association in the spike of MHV-JHM is less stable than that of MHV-A59 and that the lability of the MHV-JHM spike helps to promote RIS in vitro (20). It has been suggested that the labile interaction between S1 and S2 of MHV-JHM spike permits a spontaneous conformational change in S that exposes the fusion machinery of S2 and leads to cell-to-cell fusion without the need for binding of S1 to mCEACAM1a. For virions that are closely apposed to a cell membrane, the instability of the S1/S2 interaction might rarely allow mCEACAM1a-independent infection of a cell in the brain. Once a single cell is infected, the infection could spread throughout the CNS by fusion of infected cells with receptor-negative cells, as occurs in vitro (13, 20, 26, 27, 30, 43). The unstable S1/S2 association of MHV-JHM correlates with increased neurovirulence, as tissue culture-adapted variants that have increased stability of S1/S2 have decreased neurovirulence (10, 30, 32). These studies demonstrate that the unstable interaction between S1 and S2 of the spike of MHV-JHM

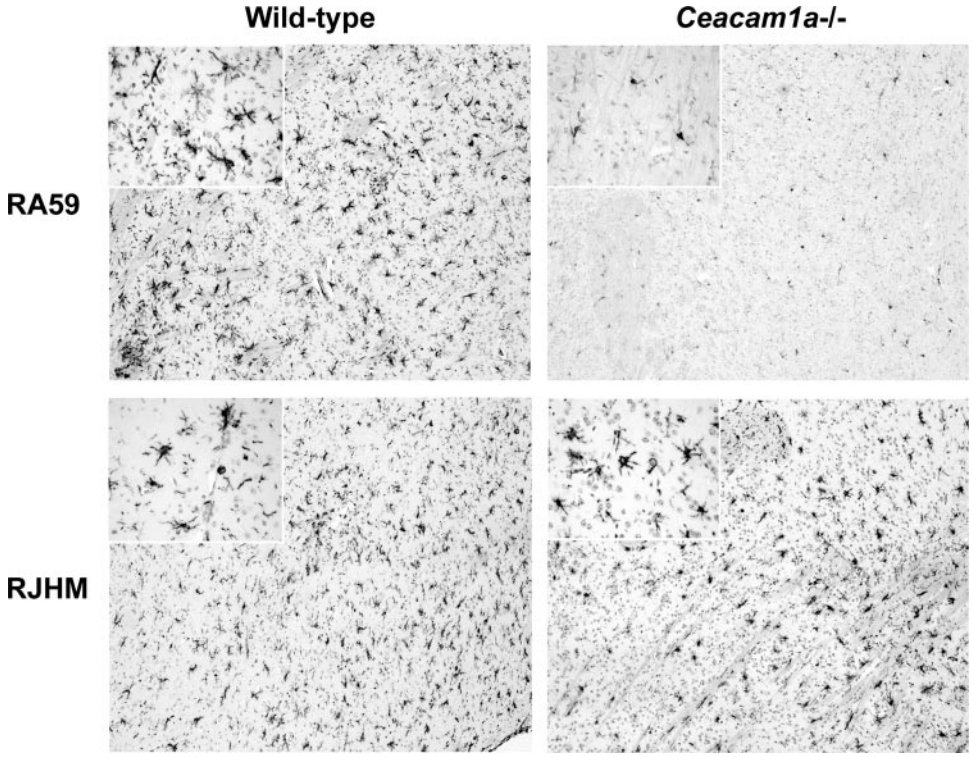


FIG. 5. Microgliosis in brain sections from wild-type C57BL/6 and *Ceacam1a*$^{-/-}$ mice infected with RA59 or RJHM. Brain sections of mice inoculated with RA59 or RJHM were analyzed by immunolabeling of microglial marker Iba1. Microgliosis, defined as increases in the number and size of microglia, was seen in wild-type mice that were inoculated with either RA59 or RJHM and in *Ceacam1a*$^{-/-}$ mice inoculated with RJHM but not RA59. Actual magnifications: large panels, ×20; insets, ×100 (to better illustrate the characteristic changes in cell morphology).

promotes RIS and increased neurovirulence. Whether the labile S1/S2 interaction of MHV-JHM spike is required for infection of *Ceacam1a*$^{-/-}$ mice is unknown.

MHV-JHM, but not MHV-A59, may use an alternative receptor, albeit inefficiently, in the CNS. Other murine proteins in the CEA family have been shown to function as receptors for strains of MHV in vitro (5, 7, 29, 50). mCEACAM1a and mCEACAM1b are encoded by alleles of the *Ceacam1* gene, and mCEACAM2 is encoded by another gene. mCEACAM1b and mCEACAM2 proteins can function as receptors for MHV-A59 and MHV-JHM when expressed at high levels in vitro (7, 29, 50). The CEA-related, pregnancy-specific glycoprotein (bCEA; PSG16) is expressed in the brains of C57BL/6 mice, and in vitro this protein can serve inefficiently as a receptor for MHV-A59 but not for MHV-JHM (5). The possible contributions of CEA-related or other, unrelated proteins in the brain as receptors for MHV-JHM are not known. If an alternative receptor is used by MHV-JHM, why is it not used by MHV-A59? Although the spike glycoproteins of MHV-JHM and MHV-A59 are very similar, they differ significantly in the hypervariable region and receptor binding domain of S1. Because receptor usage can be determined by a small number of specific amino acids, it is possible that MHV-A59 and MHV-JHM could use different receptors despite the similarity of their spike glycoproteins. Alternatively, dissociation of S1 and S2 of MHV-JHM, but not MHV-A59, could reveal an occluded binding site for an alternative receptor.

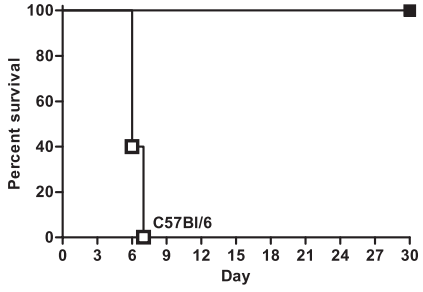


FIG. 6. Survival of wild-type C57BL/6 and *Ceacam1a*$^{-/-}$ mice after i.n. inoculation with RJHM. Five C57BL/6 and eight *Ceacam1a*$^{-/-}$ mice were inoculated i.n. with 10,000 PFU of RJHM. Mice were monitored for 30 days for clinical signs and euthanized if moribund. All three *Ceacam1a*$^{-/-}$ mice that were inoculated with dilution buffer alone survived (data not shown).

In some transgenic lines of mice that express the SARS coronavirus receptor human angiotensin converting enzyme 2 (hACE2), infection of the CNS contributes to rapid death (25, 44). It is possible that virus spread in the brain, where hACE2 expression is relatively low, is similar to our model of MHV spread in the brains of *Ceacam1a*$^{-/-}$ mice.

Although i.c. inoculation with 2,000 PFU of RJHM caused lethal infection of *Ceacam1a*$^{-/-}$ mice, i.n. inoculation with 10,000 PFU of RJHM did not result in disease (Fig. 1 and 6). In wild-type mice, i.n. inoculation requires 100-fold more virus than i.c. inoculation (22). It is difficult to determine whether, given a high enough dose, RJHM would replicate in the nasal

epithelium and spread transneuronally to the brain. If RJHM is indeed unable to infect *Ceacam1a*$^{-/-}$ mice by the i.n. route, there may be an alternative receptor in the brain that is not present in the nasal epithelium or mCEACAM1a-independent cell entry may be more efficient in the brain than in the nasal mucosa.

Using dual immunolabeling of viral antigen and cell type-specific markers, we found no difference in the cell types infected with RJHM and SJHM/RA59 in *Ceacam1a*$^{-/-}$ mice and those infected with RA59, RJHM, and SJHM/RA59 in wild-type mice. In all infected animals, neurons were the most abundant of the infected cells (Fig. 4). Previous studies suggested that infection of neurons is associated with increased neurovirulence of different isolates of MHV-JHM (11, 19). We also detected viral nucleocapsid protein in a small proportion of astrocytes and microglia and very rarely in oligodendrocytes (Fig. 4). Thus, mCEACAM1a-independent spread of infection is not limited by cell type. Although there was no difference between the proportions of cell types infected by RJHM and SJHM/RA59, RJHM infection was more widespread in the brain than SJHM/RA59 infection. We saw microgliosis in the brains of all infected animals, including wild-type C57BL/6 mice infected with RA59, RJHM, or SJHM/RA59 and *Ceacam1a*$^{-/-}$ mice infected with RJHM or SJHM/RA59 (Fig. 5). Microglia/macrophages expressing Iba1 were present in increased numbers and were much larger, presumably activated, in infected animals. These Iba1-positive cells may have been recruited into the brain, or they might result from proliferation of resident brain microglia, or both. Macrophage infiltration into the CNS and activation of resident microglia are known to occur during MHV-JHM-induced acute encephalitis and chronic demyelination (14, 21, 47, 48). We also observed a decrease in oligodendrocyte numbers in infected animals by day 7, which could be due either to killing directly by virus infection or to damage to oligodendrocytes caused by toxic molecules, such as cytokines, nitric oxide, and complement proteins, that are secreted by infected microglia (40, 47). Our observations demonstrate that disruption of the *Ceacam1a* gene does not alter the innate response of microglia/macrophages to infection with MHV. Future studies will address the impact of *Ceacam1a* disruption on other aspects of innate and acquired immunity.

MHV-A59 and MHV-JHM differ significantly in neurovirulence in wild-type mice, which is substantiated by a difference in LD_{50} and spread in the CNS after i.c. inoculation. In vitro studies have proposed that mCEACAM1a-independent spread by MHV-JHM may account for these differences in neurovirulence. Our studies of *Ceacam1a*$^{-/-}$ mice have shown definitively that MHV-JHM can infect and spread in the brain in the absence of mCEACAM1a. mCEACAM1a-independent infection and spread by MHV-JHM, but not MHV-A59, is an important difference between these virus strains that probably contributes to the greater neurovirulence of MHV-JHM.

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