Interaction of Mouse Hepatitis Virus (MHV) Spike Glycoprotein with Receptor Glycoprotein MHVR Is Required for Infection with an MHV Strain That Expresses the Hemagglutinin-Esterase Glycoprotein

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In addition to the spike (S) glycoprotein that binds to carcinoembryonic antigen-related receptors on the host cell membrane, some strains of mouse coronavirus (mouse hepatitis virus [MHV]) express a hemagglutinin esterase (HE) glycoprotein with hemagglutinating and acetylerase activity. Virions of strains that do not express HE, such as MHV-A59, can infect mouse fibroblasts in vitro, showing that the HE glycoprotein is not required for infection of these cells. The present work was done to study whether interaction of the HE glycoprotein with carbohydrate moieties could lead to virus entry and infection in the absence of interaction of the S glycoprotein with its receptor glycoprotein, MHVR. The DVIM strain of MHV expresses large amounts of HE glycoprotein, as shown by hemadsorption, acetylerase activity, and immunoreactivity with antibodies directed against the HE glycoprotein of bovine coronavirus. A monoclonal anti-MHVR antibody, MAb-CC1, blocks binding of virus S glycoprotein to MHVR and blocks infection of MHV strains that do not express HE. MAb-CC1 also prevented MHV-DVIM infection of mouse DBT cells and primary mouse glial cell cultures. Although MDCK-I cells express O-acetylated sialic acid residues on their plasma membranes, these canine cells were resistant to infection with MHV-A59 and MHV-DVIM. Transfection of MDCK-I cells with MHVR cDNA made them susceptible to infection with MHV-A59 and MHV-DVIM. Thus, the HE glycoprotein of an MHV strain did not lead to infection of cultured murine neural cells or of nonmurine cells that express the carbohydrate ligand of the HE glycoprotein. Therefore, interaction of the spike glycoprotein of MHV with its carcinoembryonic antigen-related receptor glycoprotein is required for infectivity of MHV strains whether or not they express the HE glycoprotein.

Coronaviruses are large, enveloped viruses with a plus-strand RNA genome that is 28 to 32 kb in length. The structural proteins of all coronaviruses include a nucleocapsid protein (N), a membrane glycoprotein (M), and a glycoprotein (S) that forms the large spikes or peplomers characteristic of coronaviruses (17, 32, 35). The S glycoproteins of murine coronaviruses or mouse hepatitis viruses (MHV) bind to cell membrane receptors that are isoforms of the murine biliary glycoprotein, in the carcinoembryonic antigen (CEA) subfamily of the immunoglobulin (Ig) superfamily (2, 3, 47). S also mediates fusion of the viral envelope with cellular membranes (24), leading to virus entry and uncoating in murine cells. Expression of recombinant receptor glycoproteins (called MHVR or mmCGM2) in cells of nonmurine origin makes the cells susceptible to MHV infection (2, 3).

Some strains of murine coronavirus, such as MHV-A59, express six subgenomic mRNAs, whereas others may express an additional mRNA, called mRNA 2-1, that encodes at its 5' end a hemagglutinin-esterase glycoprotein (HE) (31, 35). In MHV-A59, the subgenomic mRNA 2-1 is not transcribed (49), and there is no translation initiation codon in

the open reading frame for HE (19). Thus, cells infected with MHV-A59 do not express HE glycoprotein, and MHV-A59 virions lack HE. MHV-JHM isolates express variable levels of HE mRNA and glycoprotein depending on the passage history of the virus, which affects the number of copies of a pentanucleotide sequence at the 3' end of the leader RNA (20, 43, 49). MHV-DVIM expresses a 69-kDa glycoprotein and hemagglutinates mouse erythrocytes (39, 41). In bovine coronavirus (BCV), both surface glycoproteins S and HE have hemagglutinating activity and recognize *N*-acetyl-9-*O*-acetylneuraminic acid (Neu5,9Ac₂) residues as receptor determinants on cultured cells (26, 27). Influenza C viruses express only one surface glycoprotein, HEF, which is related to the HE of MHV (19) and, like the HE of BCV, displays acetylerase activity and binds to Neu5,9Ac₂ moieties on cell membranes (9, 25, 42).

The purpose of this research was to determine whether binding of the HE glycoprotein of MHV to O-acetylated sialic acid-containing receptors can lead to infection independent of the interaction of the S glycoprotein with CEA-related receptor glycoproteins. We examined the role of HE on MHV infectivity when binding of S to MHVR was blocked by treatment of murine cells with anti-receptor monoclonal antibody (MAb) MAb-CC1 and on nonmurine cells expressing O-acetylated sialic acids with and without transfection with MHVR cDNA. The data show that even for HE-expressing MHV

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virions, interaction of the S glycoprotein with its receptor was required for infection.

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MATERIALS AND METHODS

Cell cultures. DBT cells were established from a brain tumor in a CDF1 mouse inoculated intracerebrally with Rous sarcoma virus (11). L2 cells were derived from C3H murine fibroblasts, and 17C11 cells were derived from BALB/c 3T3 cells (37). Human rectal tumor cells (HRT-18) were kindly provided by D. Brian, University of Tennessee, Knoxville. BHK-21 baby hamster kidney fibroblasts (purchased from the American Type Culture Collection, Rockville, Md.) were transfected with MHVR cDNA subcloned in *HindIII-EagI* sites of pRSVneo (3). Madin-Darby canine kidney (MDCK I) cells (18) were transfected with MHVR cDNA as described in the following section. All cell lines were propagated in Dulbecco's modified Eagle's minimal essential medium with high glucose, 10% fetal bovine serum, and 2% penicillin-streptomycin-amphotericin B mixture (Gibco BRL, Grand Island, N.Y.).

Primary brain cultures were prepared from 2-day-old C57BL/6 mice as described by Dyer and Benjamins (4). After dissecting off the meninges, the cerebral hemispheres were digested for 10 min at 37°C in Eagle's minimal essential medium containing 0.25% trypsin (Gibco BRL) and 10 µg of DNase (Sigma, St. Louis, Mo.) per ml; centrifuged for 10 min at 150 × g; passed through a 25-gauge needle and a 60-µm sieve; centrifuged for 10 min at 150 × g; resuspended in Dulbecco's modified Eagle's minimal essential medium containing 10% fetal calf serum, 25 µg of gentamicin per ml, and 1 mM sodium pyruvate; and plated on poly-L-lysine (Sigma)-coated coverslips. Cells were refed every 3 days and used for experiments at 12 days after plating.

Stable expression of the MHV receptor in MDCK I cells. To obtain an MDCK I cell line stably expressing the MHV receptor, we constructed the expression plasmid pMHVR2. For this purpose, we used the vector pSV2NeoLAC Z⁻, derived from pSV2neo (Clontech, Palo Alto, Calif.) by inserting into its *BamHI* site a 4.3-kbp *BglII* fragment containing the β-galactosidase gene flanked by the cytomegalovirus immediate-early promoter and the simian virus 40 polyadenylation signal. To obtain pMHVR2, the β-galactosidase gene was cut out with *NciI* and replaced with the MHVR gene by cloning the 2.6-kbp *HindIII-EagI* fragment (blunt ended with Klenow DNA polymerase) of pMHVR1 (3) into the Klenow-treated vector. pMHVR2 contains the neomycin resistance gene and the MHV receptor gene under the control of the early simian virus 40 and the cytomegalovirus immediate-early promoter, respectively. Both genes are provided with a simian virus 40 polyadenylation signal. pMHVR2 was transfected into MDCK I cells by the calcium phosphate precipitation technique (7). G418-resistant colonies were propagated and tested for MHVR expression by their susceptibility to MHV-A59 infection. In most of these colonies, 20 to 30% of cells were susceptible to MHV.

Viruses. MHV-DVIM grown on DBT cells was provided by Dr. Maru, Shionogi Research Laboratories, Osaka, Japan. MHV-A59 grown on 17C11 cells was provided by L. Sturman, State Department of Health, Albany, N.Y. MHV-JHM grown on 17C11 was obtained from the American Type Culture Collection. BCV grown on HRT-18 cells was provided by D. Brian.

MHV strains and BCV were purified by equilibrium sucrose density gradient ultracentrifugation at pH 6.0 as previously described for MHV-A59 (38).

Antibodies. For immunoblot analysis, antibody to MHV-DVIM was obtained from convalescent mice. Goat antibody to the S glycoprotein of MHV-A59 was raised by immunization with gradient-purified spikes from detergent-disrupted virions (38). Rabbit antibody to the HE glycoprotein of BCV and rabbit preimmune serum were kindly provided by D. Brian.

MAbs used in the receptor blockade experiments were hybridoma culture supernatants of anti-MHVR, MAb-CC1 (48), and a control MAb of the same IgG1 isotype directed against an irrelevant protein (MAb-CT).

For immunofluorescent labeling of MHV antigens, mouse convalescent-phase anti-MHV-DVIM or anti-MHV-A59 antibody was detected with rhodamine-labeled goat anti-mouse IgG (Cappel, Durham, N.C.) and rabbit antiserum directed against sucrose density gradient-purified MHV-A59 virions with fluorescein-labeled donkey anti-rabbit IgG (Jackson ImmunoResearch, West Grove, Pa.). For labeling of glial cell antigens, rabbit polyclonal anti-galactocerebroside (1) was detected with fluorescein-labeled donkey anti-rabbit IgG (Jackson ImmunoResearch), O4, a mouse monoclonal IgM, provided by I. Sommer, Southern General Hospital, Glasgow, Scotland (34), was detected with rhodamine-labeled goat anti-mouse IgM (Jackson ImmunoResearch); and rat monoclonal anti-glial fibrillary acidic protein IgG 2A (GFAP), provided by V. Lee, University of Pennsylvania School of Medicine, Philadelphia (18), was detected with biotinylated donkey anti-rat IgG (Jackson ImmunoResearch) followed by streptavidin-conjugated 7-amino-4-coumarin-3-acetic acid (Molecular Probes, Eugene, Oreg.).

Hemadsorption assay. Hemadsorption tests were performed on MHV-infected DBT cell monolayers grown on 60-mm tissue culture plates. DBT cells were inoculated with 1 ml of culture medium or with MHV-A59 (multiplicity of inoculation [MOI], 1 PFU per cell) or MHV-DVIM (MOI, 1 PFU per cell) and incubated for 1 h at 37°C. Inocula were removed, and cells were refed and incubated at 37°C. After syncytia developed (20 h postinoculation [p.i.] for

MHV-A59, and 26 h p.i. for MHV-DVIM), a suspension of 0.4% mouse erythrocytes in phosphate-buffered saline (PBS) was allowed to adsorb for 2 h at 4°C. Monolayers were fixed with cold 2% glutaraldehyde in PBS for 15 min, and unattached erythrocytes were removed with four gentle washes of 5 ml of PBS at 4°C.

Acetylesterase assay. Acetylesterase assays were done as previously described (46). Gradient-purified virions (5 µg) were incubated at room temperature in 1 ml of PBS containing 1 mM *p*-nitrophenyl acetate (Sigma). Hydrolysis of the substrate was measured at 400 nm with a Beckman DU-7 spectrophotometer.

Immunoblot analysis. Proteins from purified virions (6 µg per well) were solubilized with 0.0625 M Tris HCl (pH 6.8)–1% SDS–10% glycerol–0.01% bromophenol blue with or without 5% β-mercaptoethanol, treated at 100°C for 5 min, separated by SDS-polyacrylamide gel electrophoresis (PAGE) on 8% gels (16), and transferred to nitrocellulose. After blocking with 2% bovine serum albumin (BSA) in dilution buffer (TNE; 50 mM Tris HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 0.05% Tween 20, 0.1% BSA), nitrocellulose sheets were incubated for 1 h with antisera or control sera used at the following dilutions in TNE: anti-MHV-DVIM and normal mouse serum, 1:100; anti-S and goat preimmune serum, 1:25; and anti-HE and rabbit preimmune serum, 1:250. Bound antibody was detected by autoradiography after incubation with radioiodinated staphylococcal protein A (Dupont NEN, Wilmington, Del.).

Receptor blockade experiments. Cells in 96-well microtiter plates were incubated with 100 µl of serial twofold dilutions of MAb-CC1 or control MAb-CT in culture medium per well for 1 h at 37°C. Antibodies were removed, and the cells were challenged with 10⁵ PFU of virus per well in 100 µl of culture medium for 1 h at 37°C. The inocula were removed, and cultures were incubated at 37°C with fresh medium. When cytopathic effects were detected (16 h for MHV-A59 and 36 h for MHV-DVIM), the MTT colorimetric assay (Chemicon, Temecula, Calif.) was performed to measure cell survival following manufacturer's instructions. The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] substrate was cleaved in the mitochondria of living cells to yield a dark-purple formazan product, and its *A*₅₇₀ was measured on an enzyme-linked immunosorbent assay (ELISA) plate reader (Dynatech, Chantilly, Va.).

Receptor blockade experiments were also done on DBT cells, BHK cells expressing MHVR, or primary glial cell cultures grown on coverslips. Confluent cells on coverslips were pretreated with anti-receptor MAb-CC1 or control MAb-CT, at a 1:2 dilution in culture medium, for 1 h at 37°C. The cells were then challenged with inocula containing MHV-A59, MHV-DVIM, or MHV-JHM (MOI, 0.2 PFU per cell) in the presence of a 1:2 dilution of MAb supernatant. After 1 h at 37°C, the inocula were removed and fresh medium with a 1:10 dilution of MAb supernatant was added. At 10 h p.i., cells were fixed for immunofluorescent staining.

The effect of anti-receptor antibody on plaque formation on DBT cells by various MHV strains was determined by pretreating monolayers with 1 ml of anti-receptor MAb-CC1 or control MAb-CT diluted 1:2 in culture medium for 1 h at 37°C, removing the MAbs, and challenging the cells for 1 h at 37°C with 0.5 ml of each 10-fold virus dilution containing 50% MAb supernatant per plate. Inocula were removed, and monolayers were overlaid with 5 ml of an agar solution containing MAb supernatant (10% MAb-CC1 or MAb-CT in minimal essential medium with 4% fetal bovine serum, 2% phenylmethylsulfonyl fluoride, and 0.95% Noble Agar). After 48 h at 37°C, plaques were visualized by 0.01% neutral red staining.

Immunofluorescent labeling of viral and cellular antigens. At 10 or 24 h p.i., DBT or MHVR-transfected or nontransfected BHK or MDCK I cells on coverslips were washed with PBS, fixed, and stained as described by Dveksler et al. (3) with 1:100 dilutions of mouse anti-MHV-DVIM or anti-MHV-A59 convalescent-phase serum followed by a 1:100 dilution of rhodamine-labeled goat anti-mouse IgG.

At 10 h p.i., brain cells were fixed for 10 min with 2% paraformaldehyde in PBS without Ca²⁺ and Mg²⁺ and immunolabeled. The phenotypes of infected cells and the presence of viral antigens were identified by three-color immunofluorescence, which enabled simultaneous visualization of oligodendrocytes with anti-galactocerebroside, astrocytes with anti-GFAP, and MHV with anti-MHV-DVIM. A second set of cells was stained to allow the simultaneous identification of oligodendrocyte type 2 astrocyte (O-2A) progenitor cells with MAb-O4, astrocytes with anti-GFAP, and MHV antigens with anti-MHV-A59. One set of coverslips was treated with anti-galactocerebroside diluted 1:50 and then with 1:50 fluorescein-conjugated donkey anti-rabbit IgG to visualize anti-galactocerebroside. The cells were then permeabilized with 100% ethanol for 10 min at –20°C to expose internal antigens. After hydration in PBS, cells were incubated with a mixture of anti-GFAP and anti-MHV-DVIM diluted 1:50. Anti-GFAP was visualized with 1:50 biotinylated donkey anti-rat IgG and then 1:40 streptavidin-conjugated coumarin. Anti-MHV-DVIM was visualized with 1:50 rhodamine-conjugated anti-mouse IgG. Coverslips were mounted on glass slides with 20% 0.02 M Tris buffer (pH 8.2)–80% glycerol. All incubations were done at room temperature, and dilutions and washes were done with PBS.

A second set of cells was stained as described above but with 1:20 MAb-O4 visualized with 1:50 rhodamine-conjugated goat anti-mouse IgG and, after permeabilization with ethanol, a mixture of 1:50 anti-GFAP and anti-MHV-A59. Anti-GFAP was visualized as described above, and anti-MHV-A59 was visualized with 1:50 fluorescein-conjugated donkey anti-rabbit IgG. Immunolabeled

FIG. 1. Acetylase activity of gradient-purified MHV-DVIM, MHV-A59, and BCV virions. Enzyme activity from 5 μ g of virion proteins was measured by hydrolysis of *p*-nitrophenylacetate. The accumulation of *p*-nitrophenol was measured by monitoring the optical density at 400 nm (O.D.₄₀₀). Each point represents the average of three measurements, with a standard deviation of less than 5%.

cells were photographed with three filter sets for rhodamine, fluorescein, and coumarin (Zeiss Axiophot, Oberkochen, Germany).

RESULTS

Expression of S and HE glycoproteins in MHV strains.

Unlike most other MHV strains, the DVIM strain of MHV causes hemagglutination and hemadsorption of mouse erythrocytes (39, 40). MHV-DVIM expresses a 69-kDa structural protein that dimerizes to form a disulfide-linked 140-kDa glycoprotein anchored on the viral envelope (41), similar to BCV (13, 50) and HCV-OC43 (12, 51). Functional and serological studies were done to show that the 69-kDa MHV-DVIM glycoprotein is homologous to the HE glycoprotein of BCV, which causes hemadsorption in infected cells and acetylase activity in virions. When monolayer cultures of DBT cells infected with MHV-A59 or MHV-DVIM were incubated in a mouse erythrocyte suspension, hemadsorption was observed only on syncytia induced by MHV-DVIM but not on syncytia induced by MHV-A59. Incubation of the MHV-DVIM-infected cells at 4°C for 1 h with polyclonal anti-MHV-DVIM prior to addition of erythrocytes prevented hemadsorption

(data not shown). A colorimetric assay that measures the release of acetate from *p*-nitrophenyl acetate showed that gradient-purified virions of MHV-DVIM, like BCV (46), had high esterase activity and confirmed that MHV-A59 had no detectable esterase activity (Fig. 1).

The structural proteins of sucrose density gradient-purified virions of MHV-DVIM, MHV-A59, and BCV were analyzed by immunoblotting (Fig. 2A). Antiserum from convalescent mice infected with MHV-DVIM recognized five proteins in unreduced preparations of MHV-DVIM virions which correspond in size to the uncleaved S (200- to 220-kDa), HE dimer (140- to 150-kDa), cleaved S (100- to 105-kDa), N (58-kDa), and M (24-kDa) proteins (32, 35). This antiserum did not recognize unreduced S of MHV-A59 or HE of BCV. After reduction of viral proteins, anti-MHV-DVIM recognized a 70-kDa glycoprotein in MHV-DVIM but did not detect the 140- to 150-kDa glycoprotein. Antibody to the HE glycoprotein of BCV detected the unreduced dimeric form (130 to 150 kDa) as well as the reduced monomeric form (65 to 70 kDa) of HE glycoproteins in MHV-DVIM and BCV virions (Fig. 2B). Antibody to the S glycoprotein of MHV-A59 recognized the cleaved and uncleaved forms of S in unreduced virion preparations of all three viruses and in reduced MHV-A59 but only the 90- to 100-kDa forms of S in MHV-DVIM and BCV after reduction (Fig. 2C). None of the three control mouse, goat, or rabbit sera reacted with any of the viral proteins. These experiments show that MHV-DVIM expressed high levels of functional HE glycoprotein, while MHV-A59 did not express HE. These two virus strains were compared to determine the role of HE in virus infection.

Effect of receptor blockade with anti-MHVR upon infectivity of HE-expressing MHV. The anti-receptor MAb-CC1 binds to the N-terminal Ig-like domain of MHVR, the 110-kDa CEA-related MHV receptor glycoprotein that was cloned from BALB/c liver cDNA, and a 58-kDa splice variant of MHVR (3). MAb-CC1 pretreatment of the 17Cl1 line of BALB/c fibroblasts prevents infection with MHV-A59 (47), and MAb-CC1 treatment of infant mice partially protected them against MHV-A59 challenge (33). The effect of MAb-CC1 on blocking infection of cultured cells by MHV-DVIM, which expresses the HE glycoprotein, was compared with that of MHV-A59, which lacks HE. The viability of DBT cells treated either with the anti-receptor antibody, MAb-CC1, or with control antibody, MAb-CT, and then challenged with the viruses was determined

FIG. 2. Immunoblots of the structural proteins of gradient-purified MHV-DVIM, MHV-A59, and BCV. Virion proteins were detected with antiserum directed against MHV-DVIM (A), the HE (gp65) glycoprotein of BCV (B), or the S glycoprotein of MHV-A59 (C), followed by ¹²⁵I-staphylococcal protein A. Molecular mass markers (in kilodaltons) are shown on the left of each blot.

FIG. 3. Protection of DBT cells from infection with MHV-DVIM or MHV-A59 by pretreatment with anti-receptor MAb-CC1. DBT cells on 96-well plates were pretreated with twofold serial dilutions of anti-receptor MAb-CC1 or control MAb-CT. Cells were then challenged with MHV-DVIM or MHV-A59 (10^5 PFU per well) and incubated until syncytium formation was detected. Cell survival was determined by the MTT colorimetric assay at an optical density of 570 nm (O.D.₅₇₀).

by the MTT colorimetric assay (Fig. 3). Anti-receptor MAb-CC1 was equally effective in protecting the DBT cells from infection with both virus strains. MAb-CC1 also blocked formation of plaques by MHV-DVIM or MHV-A59 on DBT cells (Table 1; Fig. 4) and L2 cells (data not shown). The ability of MAb-CC1 to block early steps in virus replication leading to expression of viral antigens in the cytoplasm of infected cells at

FIG. 4. Plaque assay of MHV-DVIM and MHV-A59 in the presence and absence of anti-receptor MAb-CC1. DBT cells were pretreated with anti-receptor MAb-CC1 or control MAb-CT, challenged with MHV-DVIM or MHV-A59, and overlaid with agar containing 10% MAb-CC1 or MAb-CT supernatant. Plaques were developed 48 h p.i. The virus dilutions illustrated are as follows: MAb-CT plus MHV-DVIM, 10^{-3} ; MAb-CC1 plus MHV-DVIM, 10^{-1} ; MAb-CT plus MHV-A59, 10^{-6} ; MAb-CC1 plus MHV-A59, 10^{-1} .

10 h p.i. was tested in DBT cells challenged with MHV-DVIM, MHV-JHM, and MHV-A59 (Fig. 5). Viral antigens developed in the cytoplasm, and extensive cell fusion was induced by all three viruses in cultures treated with the control MAb but not in DBT cultures treated with MAb-CC1. Thus, the DBT line of murine brain tumor cells was completely protected from MHV infection by blockade of the MHVR receptor on the cell membrane with MAb-CC1, even with a virus such as MHV-DVIM that expresses high levels of HE.

Because strong selective processes favoring virus variants may occur in the brain (15, 22, 44) and because variants expressing a 65-kDa glycoprotein, possibly HE, have been isolated from rat primary glial cell cultures after inoculation with

FIG. 5. Inhibition of virus infection of DBT cells by treatment with anti-receptor MAb-CC1. DBT cells were pretreated with control MAb-CT or with anti-receptor MAb-CC1, incubated with 0.2 PFU of MHV-DVIM, MHV-A59, or MHV-JHM per cell and MAb-CC1 or MAb-CT supernatant, and then cultured in the presence of MAb-CC1 or MAb-CT. MHV antigens at 10 h p.i. were immunolabeled with mouse anti-MHV-DVIM antiserum.

TABLE 1. Inhibition of MHV plaque formation on DBT cells by MAb-CC1^a

MAb ^b	Virus titer ^c (PFU/ml) for:		Plaque size (mm)	
	MHV-DVIM	MHV-A59	MHV-DVIM	MHV-A59
MAb-CT	1.3×10^5	5.3×10^7	1.1 ± 0.3	2.3 ± 0.4
MAb-CC1	<7	<7	None	None

^a DBT cells were pretreated with MAb-CC1 or with MAb-CT for 1 h, challenged with virus, and incubated under agar containing MABs as described in Materials and Methods.

^b MAb-CT, control MAb directed against cholera toxin (CT); MAb-CC1, anti-receptor MAb directed against MHVR, a 110-kDa receptor for MHV.

^c The percent inhibition, measured as $100 - [100(\text{PFU per ml in MAb-CC1} / \text{PFU per ml in MAb-CT})]$, was >99.9% for both viruses.

TABLE 2. Protection of MHV-infected cells in primary C57BL/6 mouse central nervous system cultures

Detecting antibody	Antibody pretreatment of cells	No. of cells positive for viral antigen ^a		
		MHV-DVIM	MHV-A59	MHV-JHM
α MHV ^b	MAB-CT	60	1,176	384
α MHV	MAB-CC1	1	1	0

^a Primary brain cells from C57BL/6 mice were pretreated with anti-receptor MAB-CC1 or control MAB-CT and inoculated with virus (5×10^5 PFU per well of a 24-well plate) containing 50% control (MAB-CT) or anti-receptor (MAB-CC1) MAb as described in Materials and Methods. The number of positive cells represents the average number of infected cells per $63\times$ microscope field on 30 random fields for MAB-CT or 50 random fields for MAB-CC1.

^b α MHV, mouse anti-MHV-DVIM or rabbit anti-MHV-A59 serum.

wt-JHM (43), receptor blockade experiments were also carried out on mixed mouse glial cell cultures. MHV-DVIM and MHV-JHM, which express HE, and MHV-A59 were inoculated onto primary glial cell cultures pretreated with anti-receptor MAB-CC1 or control MAB-CT (Table 2; Fig. 6). The number of nuclei in MHV-infected cells at 10 h p.i. was determined by immunolabeling. Cells were also labeled with anti-

FIG. 6. Inhibition of virus infection of primary glial cells by anti-receptor MAB-CC1. Primary glial cells from 2-day-old C57BL/6 mice were cultured for 12 days and then pretreated with anti-receptor MAB-CC1 or control MAB-CT. Cells were inoculated with 0.2 PFU of MHV-DVIM, MHV-A59, or MHV-JHM per cell mixed with equal volumes of MAB-CC1 or MAB-CT hybridoma supernatant and then cultured in the presence of MAB-CC1 or MAB-CT. At 10 h p.i., MHV antigens were detected by immunofluorescent labeling.

FIG. 7. Expression of MHVR glycoprotein is required for infection of MDCK I canine cells by MHV-A59 and MHV-DVIM. MDCK I cells transfected with MHVR or parental MDCK I cells were challenged with MHV-A59 or MHV-DVIM (MOI, 0.2 PFU per cell). The cells were fixed 24 h p.i., and immunofluorescent labeling was used to detect viral antigens.

bodies specific for oligodendrocytes, astrocytes, and oligodendrocyte type 2 astrocyte progenitors to identify the target cells for each virus. MHV-A59 was more efficient in infecting the glial cells than was MHV-JHM, and both were more efficient in infection than MHV-DVIM.

Simultaneous labeling with anti-neural cell antibodies and anti-MHV showed that only a small proportion of infected glial cells could be identified unequivocally. All three virus strains infected glial progenitors and oligodendrocytes, but infected astrocytes were found only in cultures inoculated with MHV-JHM (data not shown). Receptor blockade by MAB-CC1 showed that only MHVR-related proteins were functional receptors for these three MHV strains in primary mouse glial cultures and that the HE glycoprotein of MHV-DVIM did not confer a selective advantage for initial infection of these cells.

MHV-DVIM challenge of canine cells expressing recombinant MHVR. The BHK-21 line of hamster fibroblasts is resistant to infection with MHV-A59 or MHV-JHM but becomes susceptible when transfected with cDNA encoding MHVR (3). MHV-DVIM also infected BHK-21 cells transfected with MHVR but not BHK-21 cells transfected with empty plasmid (data not shown), indicating that MHVR was required for infection of BHK-21 cells with MHV-DVIM. The MDCK I line of canine kidney cells expresses Neu5,9Ac₂ and is susceptible to infection with BCV (27) and influenza C virus (9), both of which express HE glycoproteins with hemagglutination and acetyltransferase activity. MDCK I cells were susceptible to infection with MHV-A59 or MHV-DVIM only after transfection with recombinant MHVR (Fig. 7). Therefore, binding of the HE glycoprotein of MHV-DVIM to O-acetylated sialic residues on MDCK I cells could not lead to infection in the absence of binding of the viral S glycoprotein to its specific receptor glycoprotein MHVR.

DISCUSSION

MHV-DVIM binds to receptors on mouse erythrocytes, causing hemadsorption and hemagglutination, and MHV-DVIM virions have acetyltransferase activity similar to MHV-JHM (23), BCV (28, 46), HEV (28), and the HEF glycoprotein of influenza C virus (8, 45). These activities have been attributed to the HE surface glycoprotein that binds to Neu5,9Ac₂ and releases the acetyl group from position C-9 of the carbohydrate (10, 27). To determine whether the HE glycoprotein of MHV-DVIM could initiate infection of murine cells independent of an interaction of the S glycoprotein with MHVR, its CEA-related receptor glycoprotein, we blocked binding of S to MHVR with anti-receptor MAb-CC1 and challenged the cells with HE-bearing MHV strains. The anti-receptor antibody protected mouse DBT and L2 cell lines and primary glial cells from infection both with MHV-DVIM, which expresses HE, and with MHV-A59, which does not express HE (19, 29, 49). The possibility that the presence of HE on MHV strains enables these viruses to infect glial cells of different lineages was tested. Since only a small fraction of the infected cells could be identified by three-color immunofluorescence, it was not possible to assess whether the three MHV strains tested infected predominantly the same or different glial cell types, but infection of all glial phenotype was blocked by anti-receptor MAb-CC1.

To test the hypothesis that expression of HE might broaden the host range of MHV infection, affecting the ability of the virus to grow in different cell types, MDCK I cells, which express 9-O-acetylated sialic acids and are susceptible to infection by HE-bearing coronaviruses including BCV (26, 27) and hemagglutinating encephalomyelitis virus (28), were challenged with MHV-DVIM. The MDCK I cells were resistant to infection with MHV-DVIM and became susceptible only after transfection with MHVR, indicating that interaction of the viral S glycoprotein with MHVR is needed to initiate infection. These results show that binding of HE to cell membrane molecules containing Neu5,9Ac₂ is not sufficient to initiate infection of mouse and canine kidney cell lines or primary mouse brain cell cultures.

Although MHV-JHM variants expressing HE were selected by passage on cultured rat neural cells (43) and on the DBT cell line established from a mouse brain tumor (29), MHV-DVIM did not appear to have any advantage over MHV-A59 for replication in mouse primary glial cell cultures. Because the level of expression of HE varies among different MHV isolates, it has been proposed that the HE of MHV may be an accessory protein without an indispensable function in replication (49). In contrast, elimination of Neu5,9Ac₂ on MDCK I cells greatly decreases their susceptibility to BCV (27). This effect could be mediated either by the HE or S glycoproteins of BCV, both of which bind to Neu5,9Ac₂ (26). Possibly, the esterase activity of HE aids MHV-DVIM in its release from infected cells (14). Alternatively, HE may play a role in cell-to-cell spread of virus infection in the central nervous system. Unlike initial infection of cells by virions, the cell-to-cell spread of MHV-JHM in BHK or DBT cell monolayers does not require MHVR (6).

It is becoming apparent that interactions of viruses with plasma membranes sometimes involve a coordinated series of steps rather than a single step in which virus attachment protein binds to a specific receptor glycoprotein. For example, herpes simplex virus binds initially to cell surface heparan sulfate glycosaminoglycans and then initiates infection by a series of subsequent binding and penetration steps which depend on different viral glycoproteins (5, 21, 30, 36). The data in this paper suggest that a similar cascade of events occurs at the

membranes of cells inoculated with mouse coronavirus strains that express the HE glycoprotein. While HE could serve to concentrate virus on cell membranes that express Neu5,9Ac₂ moieties, virus entry is dependent on interaction of the viral spike glycoprotein with a CEA-related receptor such as MHVR.

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REFERENCES

- Benjamins, J. A., R. E. Callahan, I. N. Montgomery, D. M. Studzinski, and C. A. Dyer. 1987. Production and characterization of high titer antibodies to galactocerebroside. *J. Neuroimmunol.* **14**:325-338.
- Dveksler, G. S., C. W. Diefenbach, C. B. Cardellicchio, K. McCuaig, M. N. Pensiero, G. S. Jiang, N. Beauchemin, and K. V. Holmes. 1993. Several members of the mouse carcinoembryonic antigen-related glycoprotein family are functional receptors for the coronavirus mouse hepatitis virus-A59. *J. Virol.* **67**:1-8.
- Dveksler, G. S., M. N. Pensiero, C. B. Cardellicchio, R. K. Williams, G. S. Jiang, K. V. Holmes, and C. W. Diefenbach. 1991. Cloning of the mouse hepatitis virus (MHV) receptor: expression in human and hamster cell lines confers susceptibility to MHV. *J. Virol.* **65**:6881-6891.
- Dyer, C. A., and J. A. Benjamins. 1988. Redistribution and internalization of antibodies to galactocerebroside by oligodendroglia. *J. Neurosci.* **8**:883-891.
- Fuller, A. O., and W. C. Lee. 1992. Herpes simplex virus type 1 entry through a cascade of virus-cell interactions requires different roles of gD and gH in penetration. *J. Virol.* **66**:5002-5012.
- Gallagher, T. M., M. J. Buchmeier, and S. Perlman. 1992. Cell receptor-independent infection by a neurotropic murine coronavirus. *Virology* **191**:517-522.
- Graham, F. L., and A. J. van der Eb. 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* **52**:456-467.
- Herrler, G., I. Dürkop, H. Becht, and H. D. Klenk. 1988. The glycoprotein of influenza C virus is the haemagglutinin, esterase and fusion factor. *J. Gen. Virol.* **69**:839-846.
- Herrler, G., and H. D. Klenk. 1987. The surface receptor is a major determinant of the cell tropism of influenza C virus. *Virology* **159**:102-108.
- Herrler, G., R. Rott, H.-D. Klenk, P. Müller, A. K. Shukla, and R. Schauer. 1985. The receptor-destroying enzyme of influenza C virus is neuraminidase-O-acetyltransferase. *EMBO J.* **4**:1503-1506.
- Hirano, N., K. Fujiwara, S. Hino, and M. Matumoto. 1974. Replication and plaque formation of mouse hepatitis virus (MHV-2) in mouse cell line DBT culture. *Arch. Gesamte Virusforsch.* **44**:298-302.
- Hogue, B., and D. A. Brian. 1986. Structural proteins of human respiratory coronavirus OC43. *Virus Res.* **5**:131-144.
- King, B., and D. A. Brian. 1982. Bovine coronavirus structural proteins. *J. Virol.* **42**:700-707.
- Kruse, J., R. Mailhammer, H. Wernecke, A. Faissner, I. Sommer, C. Goridis, and M. Schachner. 1984. Neural cell adhesion molecules and myelin-associated glycoprotein share a common carbohydrate moiety recognized by monoclonal antibodies L2 and HNK-1. *Nature (London)* **311**:153-155.
- La Monica, N., L. R. Banner, V. L. Morris, and M. M. Lai. 1991. Localization of extensive deletions in the structural genes of two neurotropic variants of murine coronavirus JHM. *Virology* **182**:883-888.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**:680-685.
- Lai, M. M. 1990. Coronavirus: organization, replication and expression of genome. *Annu. Rev. Microbiol.* **44**:303-333.
- Lee, V. M., C. D. Page, H. L. Wu, and W. W. Schlaepfer. 1984. Monoclonal antibodies to gel-excised glial filament protein and their reactivities with other intermediate filament proteins. *J. Neurochem.* **42**:25-32.
- Luytjes, W., P. J. Bredenbeek, A. F. Noten, M. C. Horzinek, and W. J. Spaan. 1988. Sequence of mouse hepatitis virus A59 mRNA 2: indications for RNA recombination between coronaviruses and influenza C virus. *Virology* **166**:415-422.
- Makino, S., and M. M. Lai. 1989. Evolution of the 5'-end of genomic RNA of murine coronaviruses during passages in vitro. *Virology* **169**:227-232.
- McClain, D. S., and A. O. Fuller. 1994. Cell-specific kinetics and efficiency of herpes simplex virus type 1 entry are determined by two distinct phases of attachment. *Virology* **198**:690-702.
- Morris, V. L., C. Tieszer, J. Mackinnon, and D. Percy. 1989. Characterization of coronavirus JHM variants isolated from Wistar Furth rats with a viral-induced demyelinating disease. *Virology* **169**:127-136.
- Pfleiderer, M., E. Routledge, G. Herrler, and S. G. Siddell. 1991. High level

- transient expression of the murine coronavirus haemagglutinin-esterase. *J. Gen. Virol.* **72**:1309–1315.
24. Pfeleiderer, M., E. Routledge, and S. G. Siddell. 1990. Functional analysis of the coronavirus MHV-JHM surface glycoprotein in vaccinia virus recombinants, p. 21–31. In D. Cavanagh and T. D. K. Brown (ed.), *Coronaviruses and their diseases*. Plenum Publishing Corp., New York.
 25. Rogers, G. N., G. Herrler, J. C. Paulson, and H. D. Klenk. 1986. Influenza C virus uses 9-O-acetyl-N-acetylneuraminic acid as a high affinity receptor determinant for attachment to cells. *J. Biol. Chem.* **261**:5947–5951.
 26. Schultze, B., H. J. Gross, R. Brossmer, and G. Herrler. 1991. The S protein of bovine coronavirus is a hemagglutinin recognizing 9-O-acetylated sialic acid as a receptor determinant. *J. Virol.* **65**:6232–6237.
 27. Schultze, B., and G. Herrler. 1992. Bovine coronavirus uses N-acetyl-9-O-acetylneuraminic acid as a receptor determinant to initiate the infection of cultured cells. *J. Gen. Virol.* **73**:901–906.
 28. Schultze, B., K. Wahn, H. D. Klenk, and G. Herrler. 1991. Isolated HE-protein from hemagglutinating encephalomyelitis virus and bovine coronavirus has receptor-destroying and receptor-binding activity. *Virology* **180**: 221–228.
 29. Shieh, C. K., H. J. Lee, K. Yokomori, N. La Monica, S. Makino, and M. M. Lai. 1989. Identification of a new transcriptional initiation site and the corresponding functional gene 2b in the murine coronavirus RNA genome. *J. Virol.* **63**:3729–3736.
 30. Shieh, M. T., D. WuDunn, R. I. Montgomery, J. D. Esko, and P. G. Spear. 1992. Cell surface receptors for herpes simplex virus are heparan sulfate proteoglycans. *J. Cell Biol.* **116**:1273–1281.
 31. Siddell, S. 1983. Coronavirus JHM: coding assignments of subgenomic mRNAs. *J. Gen. Virol.* **64**:113–125.
 32. Siddell, S., H. Wege, and V. ter Meulen. 1983. The biology of coronaviruses. *J. Gen. Virol.* **64**:761–776.
 33. Smith, A. L., C. B. Cardellicchio, D. F. Winograd, M. S. deSouza, S. W. Barthold, and K. V. Holmes. 1991. Monoclonal antibody to the receptor for murine coronavirus MHV-A59 inhibits virus replication in vivo. *J. Infect. Dis.* **163**:879–882.
 34. Sommer, I., and M. Schachner. 1981. Monoclonal antibodies (O1 to O4) to oligodendrocyte cell surfaces: an immunocytological study in the central nervous system. *Dev. Biol.* **83**:311–327.
 35. Spaan, W., D. Cavanagh, and M. C. Horzinek. 1988. Coronaviruses: structure and genome expression. *J. Gen. Virol.* **69**:2939–2952.
 36. Spear, P. G., M. T. Shieh, B. C. Herold, D. WuDunn, and T. I. Koshy. 1992. Heparan sulfate glycosaminoglycans as primary cell surface receptors for herpes simplex virus. *Adv. Exp. Med. Biol.* **313**:341–353.
 37. Sturman, L. S., and K. V. Holmes. 1977. Characterization of coronavirus. II. Glycoproteins of the viral envelope: tryptic peptide analysis. *Virology* **77**: 650–660.
 38. Sturman, L. S., K. V. Holmes, and J. Behnke. 1980. Isolation of coronavirus envelope glycoproteins and interaction with the viral nucleocapsid. *J. Virol.* **33**:449–462.
 39. Sugiyama, K., and Y. Amano. 1980. Hemagglutination and structural polypeptides of a new coronavirus associated with diarrhea in infant mice. *Arch. Virol.* **66**:95–105.
 40. Sugiyama, K., and Y. Amano. 1981. Morphological and biological properties of a new coronavirus associated with diarrhea in infant mice. *Arch. Virol.* **67**:241–251.
 41. Sugiyama, K., R. Ishikawa, and N. Fukuhara. 1986. Structural polypeptides of the murine coronavirus DVIM. *Arch. Virol.* **89**:245–254.
 42. Szepanski, S., H. J. Gross, R. Brossmer, H. D. Klenk, and G. Herrler. 1992. A single point mutation of the influenza C virus glycoprotein (HEF) changes the viral receptor-binding activity. *Virology* **188**:85–92.
 43. Taguchi, F., P. T. Massa, and V. ter Meulen. 1986. Characterization of variant virus isolated from neural cell culture after infection of mouse coronavirus JHMV. *Virology* **155**:267–270.
 44. Taguchi, F., S. G. Siddell, H. Wege, and V. ter Meulen. 1985. Characterization of a variant virus selected in rat brains after infection by coronavirus mouse hepatitis virus JHM. *J. Virol.* **54**:429–435.
 45. Vlasak, R., M. Krystal, M. Nacht, and P. Palese. 1987. The influenza C virus glycoprotein (HE) exhibits receptor-binding (hemagglutinin) and receptor-destroying (esterase) activities. *Virology* **160**:419–425.
 46. Vlasak, R., W. Luytjes, J. Leider, W. Spaan, and P. Palese. 1988. The E3 protein of bovine coronavirus is a receptor-destroying enzyme with acetyl-esterase activity. *J. Virol.* **62**:4686–4690.
 47. Williams, R. K., G.-S. Jiang, and K. V. Holmes. 1991. Receptor for mouse hepatitis virus is a member of the carcinoembryonic antigen family of glycoproteins. *Proc. Natl. Acad. Sci. USA* **88**:5533–5536.
 48. Williams, R. K., G.-S. Jiang, S. W. Snyder, M. F. Frana, and K. V. Holmes. 1990. Purification of the 110-kilodalton glycoprotein receptor for mouse hepatitis virus (MHV)-A59 from mouse liver and identification of a non-functional, homologous protein in MHV-resistant SJL/J mice. *J. Virol.* **64**: 3817–3823.
 49. Yokomori, K., L. R. Banner, and M. M. Lai. 1991. Heterogeneity of gene expression of the hemagglutinin-esterase (HE) protein of murine coronaviruses. *Virology* **183**:647–657.
 50. Zhang, X. M., K. G. Kousoulas, and J. Storz. 1991. The hemagglutinin/esterase glycoprotein of bovine coronaviruses: sequence and functional comparisons between virulent and avirulent strains. *Virology* **185**:847–852.
 51. Zhang, X. M., K. G. Kousoulas, and J. Storz. 1992. The hemagglutinin/esterase gene of human coronavirus strain OC43: phylogenetic relationships to bovine and murine coronaviruses and influenza C virus. *Virology* **186**: 318–323.