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The Murine Coronavirus Mouse Hepatitis Virus Strain A59 from Persistently Infected Murine Cells Exhibits an Extended Host Range

JEANNE H. SCHICKLI,[1] BRUCE D. ZELUS,[1] DAVID E. WENTWORTH,[1] STANLEY G. SAWICKI,[2] AND KATHRYN V. HOLMES[1,3]*

Department of Microbiology[1] and Molecular Biology Program,[3] University of Colorado Health Sciences Center, Denver, Colorado 80262, and Department of Microbiology, Medical College of Ohio, Toledo, Ohio 43699[2]

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In murine 17 Cl 1 cells persistently infected with murine coronavirus mouse hepatitis virus strain A59 (MHV-A59), expression of the virus receptor glycoprotein MHVR was markedly reduced (S. G. Sawicki, J. H. Lu, and K. V. Holmes, J. Virol. 69:5535–5543, 1995). Virus isolated from passage 600 of the persistently infected cells made smaller plaques on 17 Cl 1 cells than did MHV-A59. Unlike the parental MHV-A59, this variant virus also infected the BHK-21 (BHK) line of hamster cells. Virus plaque purified on BHK cells (MHV/BHK) grew more slowly in murine cells than did MHV-A59, and the rate of viral RNA synthesis was lower and the development of the viral nucleocapsid (N) protein was slower than those of MHV-A59. MHV/BHK was 100-fold more resistant to neutralization with the purified soluble recombinant MHV receptor glycoprotein (sMHVR) than was MHV-A59. Pretreatment of 17 Cl 1 cells with anti-MHVR monoclonal antibody CC1 protected the cells from infection with MHV-A59 but only partially protected them from infection with MHV/BHK. Thus, although MHV/BHK could still utilize MHVR as a receptor, its interactions with the receptor were significantly different from those of MHV-A59. To determine whether a hemagglutinin esterase (HE) glycoprotein that could bind the virions to 9-O-acetylated neuraminic acid moieties on the cell surface was expressed by MHV/BHK, an in situ esterase assay was used. No expression of HE activity was detected in 17 Cl 1 cells infected with MHV/BHK, suggesting that this virus, like MHV-A59, bound to cell membranes via its S glycoprotein. MHV/BHK was able to infect cell lines from many mammalian species, including murine (17 Cl 1), hamster (BHK), feline (Fcwf), bovine (MDBK), rat (RIE), monkey (Vero), and human (L132 and HeLa) cell lines. MHV/BHK could not infect dog kidney (MDCK I) or swine testis (ST) cell lines. Thus, in persistently infected murine cell lines that express very low levels of virus receptor MHVR and which also have and may express alternative virus receptors of lesser efficiency, there is a strong selective advantage for virus with altered interactions with receptor (D. S. Chen, M. Asanaka, F. S. Chen, J. E. Shively, and M. M. C. Lai, J. Virol. 71:1688–1691, 1997; D. S. Chen, M. Asanaka, K. Yokomori, F.-I. Wang, S. B. Hwang, H.-P. Li, and M. M. C. Lai, Proc. Natl. Acad. Sci. USA 92:12095–12099, 1995; P. Nedellec, G. S. Dveksler, E. Daniels, C. Turbide, B. Chow, A. A. Basile, K. V. Holmes, and N. Beauchemin, J. Virol. 68:4525–4537, 1994). Possibly, in coronavirus-infected animals, replication of the virus in tissues that express low levels of receptor might also select viruses with altered receptor recognition and extended host range.

Mouse hepatitis viruses (MHVs) are a group of coronaviruses that cause inapparent infection or diseases in mice including diarrhea, hepatitis, splenolysis, immunological dysfunction, and acute and chronic neurological disorders. MHV spreads effectively from mouse to mouse, causing frequent enzootics in colonies of laboratory mice. The virus does not spread naturally from mice to other species, although intracerebral inoculation of weanling rats with MHV-JHM can lead to demyelinating disease (42). In tissue culture, MHV strains readily infect a wide variety of murine cell lines, including spontaneously transformed BALB/c 17 Cl 1 cells, L2 cells, DBT cells, J774 cells, and NCTC 1469 cells (11, 17, 38). Infection of murine cell lines with various MHV strains causes varying degrees of cell fusion and cell death and is often followed in surviving cells by long-term persistent noncytocidal infection (4, 17, 20, 22, 24, 36).

Infection of murine cells by MHV is initiated by binding of the viral spike glycoprotein (S) on the viral envelope to a specific receptor on murine cell membranes called MHVR (Bgp1a), followed by S-mediated fusion of the viral envelope with host cell membranes (25). MHVR is a biliary glycoprotein in the carcinoembryonic antigen (CEA) family of glycoproteins (44). The receptor has four immunoglobulin (Ig)-like domains, a transmembrane domain, and a short cytoplasmic tail (15). Infection of mouse cells by MHV strains can be blocked by treatment of the cells with a monoclonal antibody (MAb CC1) directed against an epitope on the N-terminal domain of MHVR (15). Fibroblasts and epithelial cells of many murine tissues, macrophages, and B lymphocytes express MHVR, which has cell adhesion functions (also called C-CAM) (21, 29, 33, 35). Anti-MHVR MAb CC1 and MHV bind to a mouse-specific epitope of biliary glycoprotein, and it is this species specificity of virus binding that is believed to be a principal determinant of the restricted host range of MHV infection (5, 10). Transfection of MHV-resistant human, hamster, or bovine cell lines with cDNA encoding MHVR makes the cells susceptible to infection with MHV strains, including MHV-A59, MHV-JHM, and MHV-3, as indicated by development of viral proteins in the cytoplasm, release of infectious virions, and

* Corresponding author. Mailing address: Department of Microbiology, Campus Box B-175, University of Colorado Health Sciences Center, 4200 E. 9th Ave., Denver, CO 80262. Phone: (303) 315-7329. Fax: (303) 315-6785. E-mail: kathryn.holmes@uchsc.edu.

development of viral cytopathic effects including cell fusion and lysis (13, 14). Several naturally occurring splice variants of MHVR and the allelic variant Bgp1b, which is expressed in MHV-A59-resistant adult SJL/J mice, can also serve as receptors for MHV-A59 and MHV-JHM in tissue culture (12, 46). In addition, several murine proteins related to MHVR but encoded by different genes, including Bgp2 and bCEA, a pregnancy-specific glycoprotein (PSG), can serve as less efficient receptors for MHV if the recombinant glycoproteins are expressed at high levels on the cell membranes (8, 32).

In general, MHV strains do not infect cells of nonmurine species in cell culture. Recent studies also show that MHV-JHM can infect primate cells in vitro and in vivo. Cabirac and coworkers showed that the neurotropic strain MHV-JHM (also called MHV-4) can cause demyelination in owl monkeys and that recoverable infectious virus could be obtained from brain tissue of the infected owl monkeys (6, 31). Baric and coworkers (2) adapted MHV-A59 to grow in hamster cells, from a mixture of MHV-A59 and MHV-JHM viruses that was serially passaged on mixed cultures of murine 17 Cl 1 cells and BHK hamster cells. The resulting mixture of persistently infected hamster and mouse cells was then serially passaged, and a recombinant, mutant MHV that infected BHK cells as well as murine cell lines was released (2). We undertook the present study to determine whether a very species-specific coronavirus such as MHV-A59 can acquire the ability to infect cells of other species during persistent infection of murine cells alone and, if so, to analyze the mechanism(s) responsible for the selection of viruses with altered host range.

Infection of 17 Cl 1 murine cells with MHV-A59 results in the fusion and death of >95% of the cells within 24 h, and the surviving cells express markedly reduced levels of MHVR (36). These cells can be serially propagated to establish a persistently infected culture without cytopathic effects but with continuous release of infectious virions from the approximately 10 to 15% of cells in the culture that are infected at any given time (36). The persistently infected cells were passaged 600 times. Virus released from passage 600 (MHV/pi600) was found to infect BHK cells. Thus, the ability of this MHV variant to infect BHK cells evolved without prior exposure to BHK cells. The virus plaque purified on BHK cells (MHV/BHK) was capable of infecting murine, hamster, human, monkey, rat, cat, and bovine cell lines. MHV/BHK was not fusogenic in these cell lines and had a much longer replicative cycle than did MHV-A59. Anti-MHVR MAb CC1 blocked infection of 17 Cl 1 cells with wild-type MHV-A59 much better than that with MHV/BHK. Soluble MHVR neutralized wild-type MHV-A59 much better than it neutralized MHV/BHK virus from the persistently infected cells. Thus, persistent infection of mouse 17 Cl 1 cells with MHV-A59, which causes a marked reduction in expression of MHVR, selected for virus with altered interactions with MHVR and a greatly extended host range.

MATERIALS AND METHODS

Cell lines. The 17 Cl 1 line of spontaneously transformed BALB/c fibroblasts (from Lawrence Sturman, New York State Health Department, Albany, N.Y. [17]); A-72 canine tumor cells (from Leonard Binn, Walter Reed Army Institute for Research, Washington, D.C.); *Felis catus* whole fetus (Fcwf) cells (from Niels Pedersen, University of California at Davis, Davis, Calif.); rat intestinal epithelial (RIE) cells (from Don Rubin, Vanderbilt University, Nashville, Tenn.); and Madin-Darby bovine kidney (MDBK) cells, Madin-Darby canine kidney (MDCK I) cells, swine testis (ST) cells, and African green monkey kidney cells (Vero 76) (from the American Type Culture Collection [ATCC], Rockville, Md.) were propagated in Dulbecco's modified Eagle's medium (DMEM; GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% heat-inactivated fetal calf serum (FCS; Gemini Bioproducts, Inc., Calabasas, Calif.)–200 U of penicillin G sodium per ml–200 μg of streptomycin sulfate per ml–0.50 μg of amphotericin B (GIBCO Laboratories, Grand Island, N.Y.) per ml. The L132 and HeLa cell lines (from the ATCC) were propagated in Eagle's minimal essential medium supplemented with 10% heat-inactivated FCS and 10 μg of gentamicin (GIBCO Laboratories) per ml. The BHK-21 (called BHK below) line of baby hamster kidney fibroblasts (from the ATCC) and the 17 Cl 1 cells persistently infected with MHV-A59 were propagated in DMEM supplemented with either 6 or 10% FCS–5% tryptose phosphate broth–200 U of penicillin G sodium per ml–200 μg of streptomycin sulfate per ml, and 0.50 μg of amphotericin B per ml.

Viruses. The A59 strain of MHV and MHV-DVIM were propagated in 17 Cl 1 cells as previously described (17, 19). The MHV/BHK virus was derived from passage 600 of a culture of 17 Cl 1 cells persistently infected with MHV-A59 as previously described (36). Briefly, 17 Cl 1 cells were inoculated with MHV-A59 at a multiplicity of infection (MOI) of 100 PFU/cell, and surviving cells were serially passaged every 3 days for more than 600 passages. At intervals, virus was harvested from the supernatant medium, quickly frozen, and stored at −80°C. Virus from passage 600 (called pI#3p600) was plaque purified twice in 17 Cl 1 cells to yield a virus called MHV/pi600 which was found to grow in BHK cells as well as murine 17 Cl 1 cells. This virus was then serially passaged 12 times in BHK cells; fresh cells were inoculated at each passage with <0.1 PFU/cell, and the virus was harvested from the supernatant medium at 3 days postinoculation (p.i.). The virus grown in BHK cells was then plaque purified twice in BHK cells to yield a virus called MHV/BHK (summarized in Fig. 1). This virus was propagated in 17 Cl 1 cells or, for some experiments, in BHK cells, and virus infectivity was assayed by plaque assay in 17 Cl 1 cells as previously described (19, 38).

The stocks of MHV/BHK used in the experiments below were prepared by inoculating BHK cells with plaque-purified MHV/BHK at an MOI of 0.01 PFU/cell. At 48 h p.i., the supernatant medium was collected and centrifuged at 200 × *g* for 10 min to remove cell debris, frozen in a dry ice-ethanol bath, and stored at −80°C. This virus had a titer of 7×10^6 PFU/ml in 17 Cl 1 cells.

Antibodies. An immunoglobulin G1 (IgG1) mouse MAb directed against the MHV nucleocapsid protein (N) was generously provided by Julian Leibowitz (Texas A&M University, College Station, Tex.). MAb CC1, an IgG1 mouse MAb directed against the N-terminal domain of MHVR, was used for receptor blockade experiments described below. A control IgG1 mouse MAb directed against an irrelevant antigen (the B subunit of cholera toxin) was used in all immunofluorescence experiments. Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pa.) was used at a 1:400 dilution.

Time course of viral RNA synthesis. The rate of actinomycin D-resistant synthesis of virus-specific RNA was determined as described previously (37). Briefly, cells in 35-mm petri dishes were infected with MHV-A59 and viral RNA was radioactively labeled by incubating the cells for 1 h in low-pH DMEM containing 6% FCS, 20 μg of actinomycin D per ml, and 50 or 100 μCi of [^{3}H]uridine per ml. After the labeling period, the cells were rinsed with ice-cold phosphate-buffered saline (PBS) and solubilized in 5% lithium dodecyl sulfate–200 μg of proteinase K per ml–0.1 M LiCl–10 mM Tris-HCl (pH 7.4 to 7.6)–1 mM EDTA in DMEM. After the DNA was sheared through a 27-gauge needle, duplicate samples containing extracts from approximately 50,000 cells were made and precipitated with 10% trichloroacetic acid. The precipitates were collected on glass fiber filters which were dried and subjected to liquid scintillation spectroscopy.

Immunolabeling of cells infected with MHV. Cells grown on coverslips in 60-mm petri dishes were inoculated with 0.6 ml of either MHV-A59 or MHV/BHK at 3 to 5 PFU/cell. Following adsorption at 37°C for 1 h, the virus inoculum was replaced with 3 ml of fresh medium, and the cells were incubated at 37°C. At 6, 10, 17, or 24 h p.i., the cells on coverslips were washed in Dulbecco's PBS (GIBCO), fixed in acetone at −20°C for 10 min, air dried, and stored at −20°C. The fixed cells were rehydrated in PBS and incubated for 30 min with 2% normal goat serum in PBS. Nucleocapsid (N) protein was detected with anti-N MAb and FITC-labeled goat anti-mouse IgG as previously described (14). Controls that showed no immunolabeling included virus-inoculated cells incubated with a control IgG1 MAb directed against an irrelevant antigen and FITC-labeled goat anti-mouse IgG, mock-infected cells incubated with anti-N MAb and FITC-labeled goat anti-mouse IgG, and nonmurine cells inoculated with MHV-A59 and incubated with anti-N MAb and FITC-labeled goat anti-mouse IgG. Each cell line was tested in three independent experiments.

Anti-MHVR MAb CC1 receptor blockade experiments with MHV-A59 and MHV/BHK viruses. 17 Cl 1 cells in 100-mm petri dishes were pretreated with 2 ml of a 1:2 dilution of hybridoma supernatants containing either anti-MHVR MAb CC1 or a control MAb. After 1 h at 37°C, 1 ml of medium containing either MHV-A59 or MHV/BHK (3 PFU/cell) was added and the viruses were allowed to adsorb for 3 h at 37°C. The inocula were removed, and the plates were washed twice with DMEM. Then 3 ml of medium containing a 1:2 dilution of either MAb CC1 or control MAb hybridoma supernatant was added, and the cultures were incubated at 37°C. At 4, 21, and 28 h p.i., 300 μl of supernatant medium was collected and the titers of infectious virus were determined by a 50% tissue culture infective dose assay. Briefly, a 96-well microtiter plate containing 17 Cl 1 cells (85% confluency) was inoculated in triplicate with 0.1 ml of serial 10-fold dilutions (10^{-1} to 10^{-8}) of the supernatant media collected at different times postinfection. After incubation at 37°C for 2.5 days, the cells were fixed and stained with crystal violet solution (0.5% crystal violet, 0.84% NaCl, 26% etha-

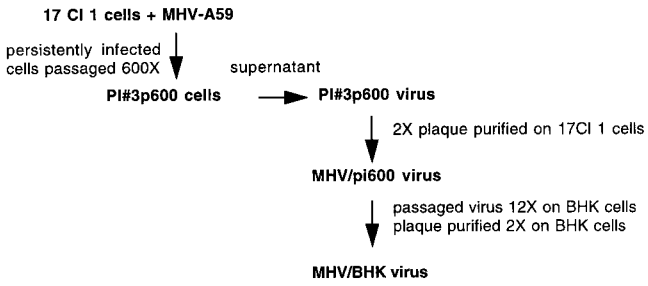

FIG. 1. Derivation of MHV/BHK virus with extended host range.

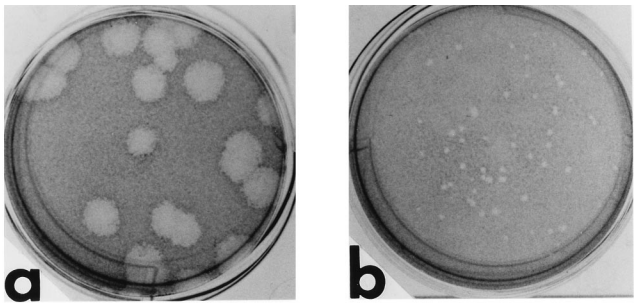

FIG. 2. Plaque morphologies of MHV-A59 and MHV/BHK in murine 17 Cl 1 cells. 17 Cl 1 cells were infected with either MHV-A59 (A) or MHV/BHK (B). Cells were stained with neutral red and photographed 3 days p.i. MHV-A59 produced plaques 4 to 5 mm in diameter while MHV/BHK produced plaques 0.5 to 1 mm in diameter.

nol, 1.85% formaldehyde). Wells containing any zones of clearing due to virus replication were recorded as positive.

Neutralization of MHV/BHK and MHV-A59 by purified soluble recombinant anchorless MHVR glycoprotein. Virus and recombinant soluble receptor (sMHVR) were serially diluted in Tris-buffered saline–TBS + 5% glycerol containing 0.1 mg of bovine serum albumin (Fraction V; Sigma) per ml. A total of 30 μl (5,000 PFU) of either MHV-A59 or MHV/BHK was mixed with 180 μl of diluted receptor and incubated at 37°C for 1 h. The virus-sMHVR mixture was diluted 10-fold with DMEM and immediately used to inoculate 17 Cl 1 cells for plaque assay. Plaques were counted 48 h p.i. (MHV-A59) or 72 h p.i. (MHV/BHK).

In situ esterase assay. Plaque assays with 10-fold serial dilutions of either MHV-A59, MHV-DVIM, or MHV/BHK were done with 17 Cl 1 cells and BHK cells in six-well plates in duplicate. Neutral red agar (0.025%) was added to one set of wells at 48 h p.i. for MHV-A59 and at 72 h p.i. for MHV-DVIM and MHV/BHK. For the esterase assay, when plaques were visible on the neutral red-stained plates, the agar was removed from the duplicate wells without neutral red. The general procedure for the in situ esterase assay has been described previously (41). Briefly, the cells were washed twice with PBS. On ice, the cells were fixed in cold formalin-acetone solution (25% formalin and 45% acetone in 0.067 M sodium phosphate buffer) for exactly 30 s. Pararosaniline was solubilized in 2 N HCl (1 g of pararosaniline, 20 ml of H_2O, 5 ml of concentrated HCl). Hexazotinized pararosaniline was prepared by mixing equal volumes of 4% sodium nitrite and solubilized pararosaniline immediately prior to use. The α-naphthyl acetate (ANA) substrate solution was prepared immediately prior to use by mixing ANA solubilized in ethylene glycol monomethyl ether with hexazotinized pararosaniline and adjusting the pH to 6.0 with 2% NaOH while stirring rapidly to minimize the particle size of any precipitate. The ANA substrate solution (1.5 ml) was added to each well. After 30 min, the cells were washed with PBS and then water and air dried. Insolubilized dark red substrate deposited in MHV-infected cells in and around the virus plaques indicated activity of a virus-encoded esterase. MHV-DVIM was the positive control for expression of the hemagglutinin esterase (HE) glycoprotein, and MHV-A59 was the negative control (19).

RESULTS

Infection of hamster cells by virus isolated from murine cells persistently infected with MHV-A59. The passage history of a culture of 17 Cl 1 cells persistently infected with MHV-A59 is outlined in Fig. 1 and has been previously described in part (36). The yield of virus remained constant between 10^6 and 10^7 PFU/ml over the 5-year period of culture. From the medium at the 600th passage of infected cells, virus (MHV/pi600) was isolated and then plaque purified three times in 17 Cl 1 cells. MHV/pi600 produced smaller plaques than did MHV-A59 (0.5 to 1 mm versus 4 to 5 mm, respectively, at 3 days p.i.). Fusion of 17 Cl 1 cells was less extensive and occurred much later for MHV/pi600 compared to MHV-A59. Large multinucleated giant cells appeared 6 to 8 h after MHV-A59 infection and 18 to 20 h after infection with MHV/pi600.

In contrast to the highly species-specific MHV-A59 virus, MHV/pi600 was able to infect BHK cells. The mutations in the virus population that enabled MHV/pi600 to infect BHK cells had been selected in persistently infected murine cells without prior exposure to BHK cells. The MHV/pi600 virus was serially passaged on fresh BHK cells 12 times at a low MOI and then plaque purified in BHK cells (Fig. 1). The virus that had been plaque purified in BHK cells was called MHV/BHK. As with MHV/pi600 virus, the plaques produced by MHV/BHK were small (0.5 to 1 mm at 3 days p.i.) on both 17 Cl 1 cells (Fig. 2) and BHK cells (data not shown).

Synthesis of MHV/pi600 and MHV/BHK viral RNA and nucleocapsid protein was delayed compared to that of wild-type MHV-A59 virus. To determine whether the observed small plaque size was the result of lower cell fusion activity of MHV/pi600 and MHV/BHK or was due to reduced viral replication, we studied the time course of infection with these viruses on 17 Cl 1 cells. The rates of actinomycin D-resistant viral RNA synthesis of both the persistent viruses MHV/pi600 and MHV/BHK were lower than that of wild-type MHV-A59 (Fig. 3). The peak of MHV/pi600 RNA synthesis occurred at 10 h, much later than the 6-h peak for MHV-A59. The peak height for viral RNA synthesized in murine cells was roughly threefold lower for MHV/pi600 than for MHV-A59 (Fig. 3). Similar results were obtained for the MHV/BHK virus. In BHK cells, MHV-A59 did not replicate at all and the rates of RNA synthesis for both MHV/pi600 and MHV/BHK were markedly lower than for the same viruses in murine cells. Infection with these viruses required more than 24 h to reach the level of RNA produced by MHV-A59 in 17 Cl 1 cells at 6 h. In Vero cells, MHV-A59 did not replicate at all, and high levels of RNA synthesis for both MHV/pi600 and MHV/BHK occurred by 10 h, which was sooner than on BHK cells. Thus, MHV/pi600 and MHV/BHK grown on BHK cells had longer replicative cycles than the parental virus but had similar replicative cycles compared to parental virus when grown on Vero cells.

Immunolabeling was used to monitor the time course of accumulation of nucleocapsid antigen in BHK cells infected with MHV/BHK virus. Coronavirus nucleocapsid protein (N) characteristically appears first in the perinuclear area and then later becomes distributed throughout the cytoplasm. In BHK cells infected with MHV/BHK, at 10 h p.i. very little N was evident and only roughly 10% of the cells were infected. An unexpected observation was that N antigen initially appeared localized in two regions on opposing sides of the nucleus. After 17 h, the N antigen had spread throughout the cytoplasm of the BHK cells in dense granules and 100% of the BHK cells were infected. After 24 h, abundant N antigen was present throughout the cytoplasm of the BHK cells and limited fusion (syncytia with only two nuclei) had occurred in less than 1% of the cells (Fig. 4). In contrast, in 17 Cl 1 cells, 100% of the cells were infected by MHV/BHK 10 h p.i. and there was moderate cell fusion. In 17 Cl 1 cells infected with the parental MHV-A59, N

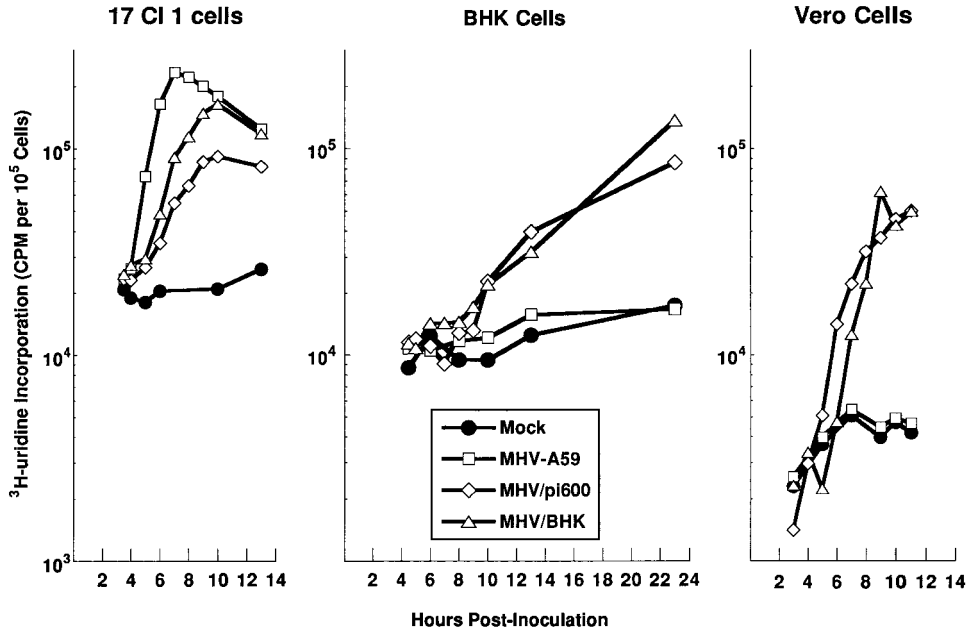

FIG. 3. Rates of virus-specific RNA synthesis in murine, hamster, and primate cells. [^{3}H]uridine was incorporated into viral RNA in actinomycin D-treated cells for 1 h before harvesting at the time points indicated as described in Materials and Methods. The rates of RNA synthesis of MHV-A59 (squares), MHV/pi600 (diamonds), and MHV/BHK (triangles) or mock-infected cells (circles) are compared in the murine 17 Cl 1 cell line, the hamster BHK cell line, and the monkey Vero 76 cell line.

antigen was detected in 100% of the cells after 6 h and there was extensive cell fusion by 10 h p.i.

Anti-MHVR MAb CC1 blockade of virus infection of 17 Cl 1 cells. Because the level of MHVR is markedly reduced in the persistently infected 17 Cl 1 cells, it was important to determine whether the variant virus isolated from these cells was still able to infect murine cells through the interaction of the viral S protein with MHVR. We therefore investigated the ability of anti-MHVR MAb CC1 to block infection of 17 Cl 1 cells by MHV/BHK. Previous studies showed that anti-MHVR MAb CC1 blocks infection by MHV-A59 (14). When 17 Cl 1 cells were pretreated for 1 h with MAb CC1 and MAb CC1 was present during virus adsorption and replication, infection with MHV-A59 was completely blocked but infection by MHV/BHK was only partially inhibited (Fig. 5).

Neutralization of viruses by soluble recombinant MHVR. Another approach we used to analyze the interaction between the S protein and MHVR quantified neutralization by soluble anchorless recombinant MHVR that contained Ig-like domains 1 to 4 (sMHVR). The infectivity of the parental MHV-A59 is effectively neutralized by incubation with sMHVR (48). If MHVR is the primary receptor on murine cells utilized by MHV/BHK, then incubation of the virus with sMHVR should block infection of 17 Cl 1 cells. Figure 6 shows that 50% of the infectivity of MHV-A59 was neutralized by <1 nM sMHVR. In contrast, MHV/BHK required more than 100 nM sMHVR

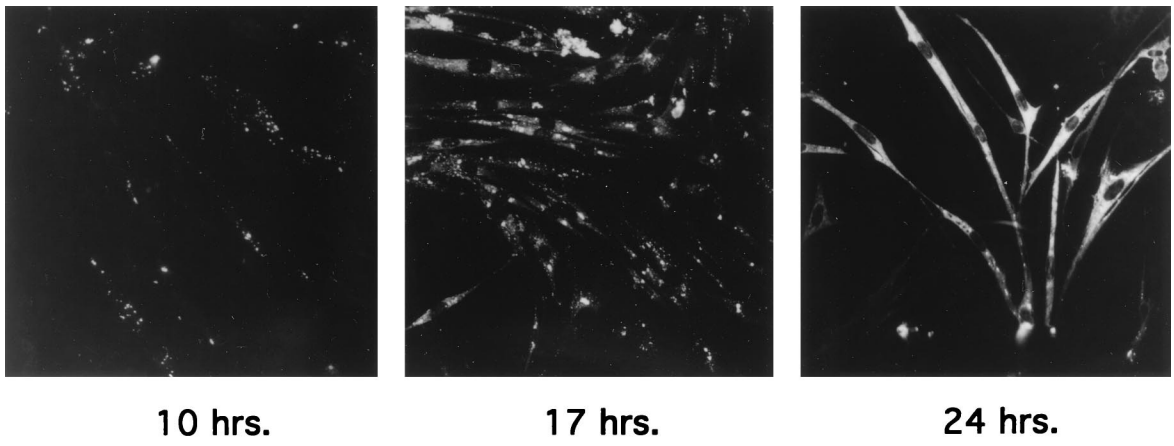

FIG. 4. Development of viral nucleocapsid antigen in the cytoplasm of hamster cells infected with MHV/BHK. BHK cells were infected with MHV/BHK at 3 PFU/cell. At 10, 17, and 24 h p.i., the cells were fixed in -20°C acetone. The presence of viral nucleocapsid in the cytoplasm of infected cells was detected by immunolabeling with mouse anti-nucleocapsid MAb and FITC-conjugated goat anti-mouse IgG. Magnification, $\times$400.

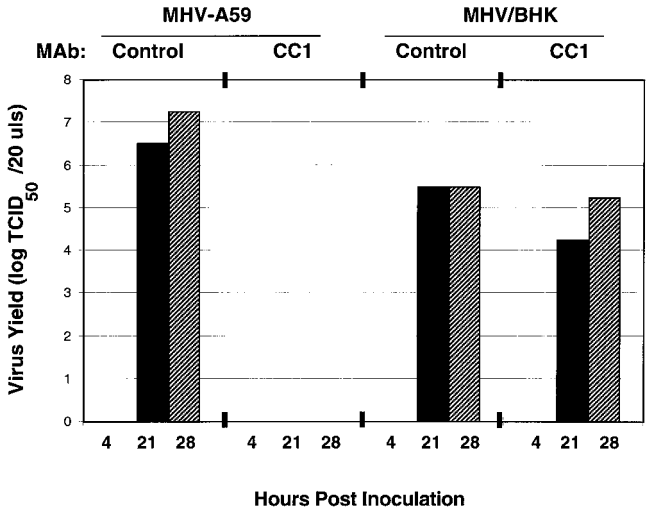

FIG. 5. Yields of MHV-A59 and MHV/BHK from murine 17 Cl 1 cells treated with anti-MHVR MAb CC1. Virus yields at 4, 21, and 28 h p.i. with MHV-A59 or MHV/BHK in the presence of anti-MHVR MAb CC1 or a control MAb were determined by 50% tissue culture infective dose ($TCID_{50}$) in 17 Cl 1 cells. Although replication of MHV-A59 was completely blocked in MAb CC1-treated cells, replication of MHV/BHK was only partially inhibited.

to neutralize only 40% of its infectivity. The observation that MHV/BHK was at least partially neutralized by high levels of sMHVR indicates that this variant virus still binds MHVR. However, the efficiency of neutralization of the variant virus by sMHVR was reduced by more than 100-fold relative to that for MHV-A59. Thus, the virus that had been selected in persistently infected murine cells that express low levels of MHVR had a markedly altered interaction with sMHVR.

Expression of HE by MHV strains. Some strains of MHV, such as MHV-JHM, express the HE glycoprotein on the viral envelope. Like the HE glycoprotein of influenza C virus, HE of MHV can bind to 9-O-acetylated neuraminic acid residues on cell membranes, possibly providing an alternative to the spike-

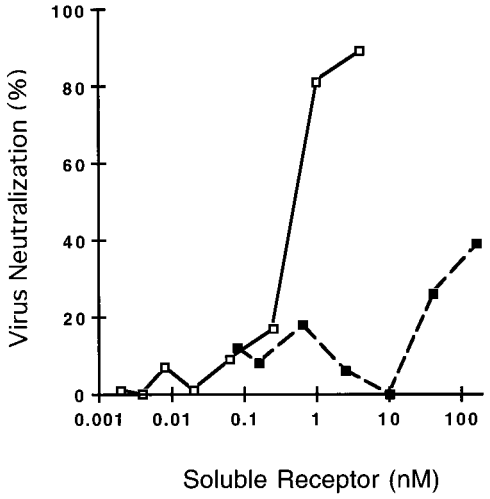

FIG. 6. Neutralization of MHV by purified soluble, anchorless recombinant MHVR (sMHVR). The viruses MHV-A59 (open symbols) and MHV/BHK (filled symbols) were incubated with varying amounts of sMHVR for 1 h at 37°C and then assayed by plaque formation on 17 Cl 1 cells. The percent neutralization was calculated relative to that for virus incubated with buffer alone.

MHVR interaction to initiate virus infection (40). All MHV strains studied to date have the HE gene in the viral genome, but the A59 strain of MHV does not express HE because of a series of three different types of mutations (19, 45). To determine if MHV/BHK had mutations that allowed expression of HE, we performed an in situ esterase assay (Fig. 7). Plaques from MHV-DVIM, which expresses HE, were positive for esterase activity, exhibiting a dark red pararosaniline precipitate in the infected cells. The number of plaques visualized by this esterase assay was equal to the number of plaques visualized by neutral red staining of duplicate plates. In contrast, no precipitate was seen in plaques from both MHV-A59 and the MHV/BHK derived from persistently infected cells. These data suggest that MHV/BHK virus does not express HE and so the virions probably interact with the cellular receptor(s) via their S glycoprotein.

Broad host range of the MHV/pi600 and MHV/BHK viruses derived from persistently infected mouse cells. Because the MHV/BHK virus derived from persistently infected 17 Cl 1 cells had altered interactions with MHVR, the receptor for the parental MHV-A59, and had also acquired the ability to infect BHK cells, we also tested whether MHV/BHK could infect cell lines from several other species. The presence of N antigen (Fig. 8) shows that MHV/BHK could infect the feline cell line Fcwf, although the parental MHV-A59 could not infect Fcwf cells. Immunolabeling showed that MHV/BHK infected cell lines of mice (17 Cl 1), hamsters (BHK), humans (L132 and HeLa), primates (Vero), cats (Fcwf), cows (MDBK) (Fig. 9), and rats (RIE) (data not shown). In contrast, MHV/BHK did not readily infect canine or pig cells; less than 1% of the canine tumor cell line (A-72) was infected, and no infection was detected in either the canine kidney (MDCK I) or the swine testis (ST) cell lines (Fig. 9). The amount of N antigen, its distribution in the cytoplasm, and the percentage of cells infected varied considerably among cell lines of different species. At 24 h p.i. with MHV/BHK at an MOI of 3 to 5 PFU/cell, 50 to 100% of HeLa and L132 cells were infected, but they expressed only a moderate level of viral protein N. At 24 h p.i., small foci of 5 to 30% of Vero and Fcwf cells were infected and these cells showed more N antigen. MHV/BHK induced cell fusion only in the murine cells and in less than 1% of the BHK cells. Vero cells infected with MHV/BHK showed one or two punctuate regions of N antigen in each nucleus. The anti-N MAb did not detect any nuclear component of uninfected Vero cells, so either N of MHV/BHK was trafficking into the nucleus or the infection induced the synthesis of a cellular nuclear protein that cross-reacted with the anti-N MAb.

To determine whether the 12 passages of MHV/BHK virus in hamster cells had resulted in selection of a virus with broader host range than that of the MHV/pi600 virus from persistently infected murine cells, we studied the ability of MHV/pi600 to infect Vero cells. Figure 3 shows that MHV/pi600 grows in Vero cells as well as does MHV/BHK virus. MHV/pi600 forms plaques on BHK cells and on Vero cells (data not shown). Thus, virus that had the ability to grow on both hamster cells and monkey cells was selected during growth of the virus in persistently infected murine cells.

DISCUSSION

17 Cl 1 mouse cells persistently infected with MHV-A59 express markedly reduced levels of the virus receptor glycoprotein MHVR, which may favor the selection of variant viruses that interact differently with cellular receptor(s) than does MHV-A59 (36). MHV/BHK replicated more slowly than wild-type MHV-A59, caused delayed and less extensive cell

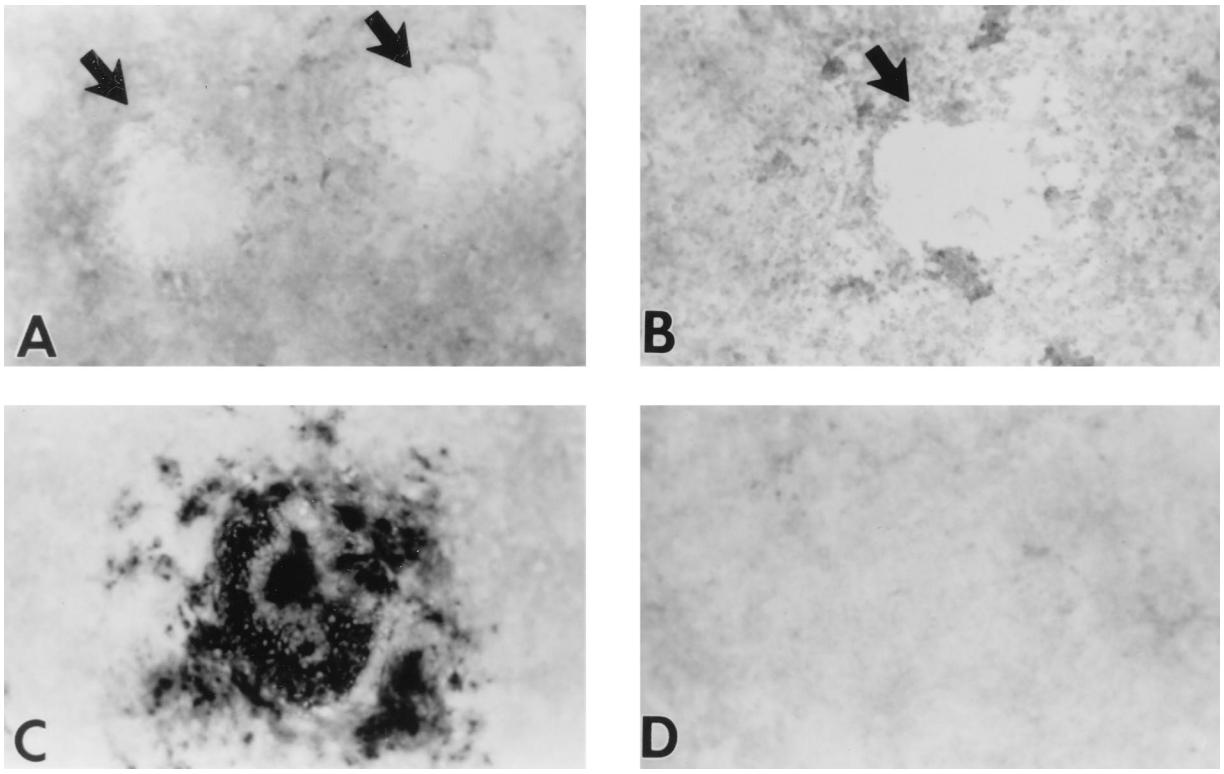

FIG. 7. Acetyl esterase activity of HE glycoprotein of different strains of MHV. Virus plaques on 17 Cl 1 cells were fixed at 48 (MHV-A59) or 72 (MHV-DVIM and MHV/BHK) h p.i. The insoluble red stain resulting from cleavage of the indicator dye by virus-encoded acetylesterase was seen in plaques of MHV-DVIM (C) but not in plaques (arrows) formed by MHV-A59 (B) or MHV/BHK (A) or in uninfected control cells (D). Magnification, ×20.

fusion, and formed smaller plaques than did wild-type MHV-A59. Growth of the mutant virus in mouse cells was not completely inhibited by anti-MHVR MAb CC1, and the virus was less efficiently neutralized by soluble recombinant MHVR than was wild-type MHV-A59. This variant virus still interacted with receptors via its S envelope glycoprotein because it did not express HE that might have bound to alternative carbohydrate receptors. These observations suggest either that the mutant virus may not utilize MHVR as its only receptor for infection of murine cells or that it may use MHVR as its receptor, but differently than wild-type MHV-A59.

We found that, unlike the parental MHV-A59, virus from passage 600 of the persistently infected murine 17 Cl 1 cells (MHV/pi600) could infect hamster cells. In hamster cells, replication of viral RNA, accumulation of viral antigens, and virus-induced cytopathic effects were considerably delayed in comparison with these processes in mouse cells. However, high yields of infectious virus were released from the hamster cells by 24 h p.i. Virus from the persistently infected murine cell culture was twice plaque purified in BHK hamster cells (MHV/BHK) and then used to inoculate cells of other species. We found that MHV/BHK could infect cells of mice, hamsters, cats, cows, rats, monkeys, and humans. Interestingly, only a small percentage of the cells in the A-72 dog cell line were infected, while the MDCK I line of dog cells was completely resistant to infection with the MHV/BHK virus. A swine testis (ST) cell line was also completely resistant to infection with MHV/BHK. Possibly these dog and pig cells fail to express an appropriate receptor for the variant virus. Alternatively, dog and pig cells may lack a putative cofactor required for initiation of infection after virus binding. Thus, virus isolated from persistently infected murine cells that express low levels of MHVR had developed a widely extended host range.

It is important to note that the MHV/pi600 from persistently infected murine cells developed the ability to infect and grow in cells of nonmurine species without any period of adaptation to those cell lines (Fig. 3). Baric and coworkers selected a host range variant of MHV (MHV-H2) that had a recombinant S glycoprotein after extended serial passages of a mixture of MHV-A59 and MHV-JHM viruses in a culture of mixed hamster and murine cells, followed by serial passages of the persistently infected cells (2). In that study, the virus was constantly exposed to both murine and hamster cells, so that mutations favoring growth in BHK cells would have had an opportunity to be selected. In contrast, MHV/pi600 was selected in persistently infected murine cells that expressed low levels of MHVR. This is a more biologically relevant model in which to study the selection of mutations that have acquired the ability to infect cells of many additional species as well as the original host.

In MHV-susceptible BALB/c mice, tissues at the portals of virus entry, such as small intestine, colon, and respiratory tract, express high levels of MHVR and other tissues such as brain and spinal cord express very low or undetectable levels of MHVR (21, 44). In acute MHV infection of susceptible mice, virus enters via the respiratory and/or alimentary tracts, in which high levels of receptor are expressed, and replicates in these tissues, releasing large amounts of virus into respiratory and enteric fluids which may readily be transmitted to other animals. In the infected mouse, virus that spreads systemically may reach other tissues that express lower levels of MHVR and possibly also alternative MHV receptors of lower efficiency

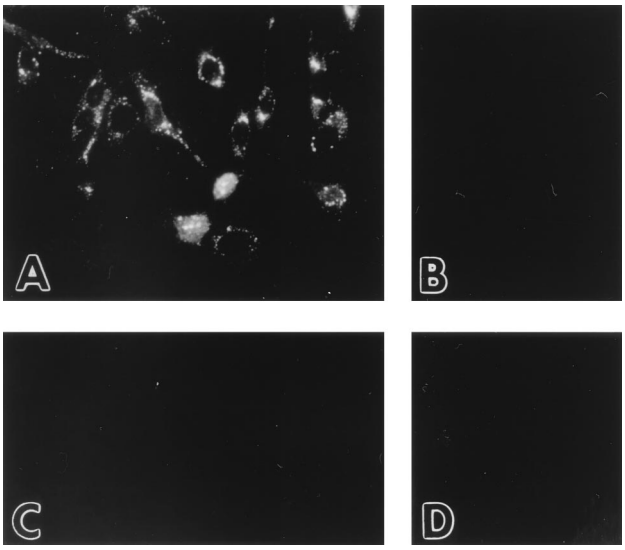

FIG. 8. Susceptibility of feline cells to infection with MHV strains. Fcwf cells were inoculated with either MHV/BHK (A and D) or MHV-A59 (B) at an MOI of 3 to 5 PFU/cell or with control medium (C). At 24 h p.i., MHV nucleocapsid protein was detected by immunolabeling in the cytoplasm of infected cells. MHV/BHK, which was derived from persistently infected murine cells, infected the feline cells, but the parental MHV-A59 did not. Cells inoculated with MHV/BHK and incubated with control MAb directed against an irrelevant antigen and FITC-labeled goat anti-mouse IgG1 (D) and sham-inoculated cells incubated with anti-N MAb and FITC-labeled anti-mouse IgG1 (C) showed no immunolabeling. Magnification, approximately ×300.

such as Bgp2 or bCEA (8, 32). Possibly in those tissues the virus could establish a persistent infection, particularly if it reaches an immunologically privileged site like the central nervous system, or if the host immune response is suppressed. As in persistently infected murine cultures, in the infected mouse virus variants may be selected in tissues that have either naturally occurring low levels of receptor expression or levels that have been reduced in response to virus infection and that also express alternative receptors of lower efficiency. Such viruses might have extended host range. However, virus from such internal organs is probably less likely to be shed and transmitted to other mice than virus produced in the respiratory or enteric tracts. Perhaps a putative receptor-variant virus from an internal organ would never be shed from the infected animal, although it might affect pathogenesis in its natural host. However, if a predator or scavenger ate the infected mouse, then virus from its internal organs might have the opportunity to infect the new species.

After a receptor-variant virus binds to and enters the cells of a new host, additional mutations are probably required for optimal adaptation to some of the host-dependent processes of virus replication that follow virus entry. For example, the synthesis of MHV/pi600 and MHV/BHK RNA is significantly faster and/or higher in murine cells than in hamster or monkey cells (Fig. 3). Possibly, continued passage of these variant viruses in hamster or monkey cells would select for further virus variants that show increased levels of viral RNA synthesis in these host cells. If such secondary mutations occur, so that infectious virus is shed before the infection can be eliminated by the immune response, or if the uninfected animal is ingested by a susceptible predator or scavenger, then the virus might be able to be transmitted in the new host species. Fortunately, viruses very rarely jump to new hosts, perhaps because an unlikely succession of mutations is needed to optimize the many host-dependent steps in the virus replicative cycle in the new host species.

The central hypothesis that has developed from data in this article is that in persistently infected cells in vitro or, perhaps, in tissues with low levels of virus receptor that also express alternative receptors of lower efficiency, virus with mutant receptor interactions may be selected. Such a virus might be able to initiate the multistep process of adapting the virus to replication in a new host species.

Emerging viral diseases are frequently, but not always, caused by viruses which readily cause persistent infections in

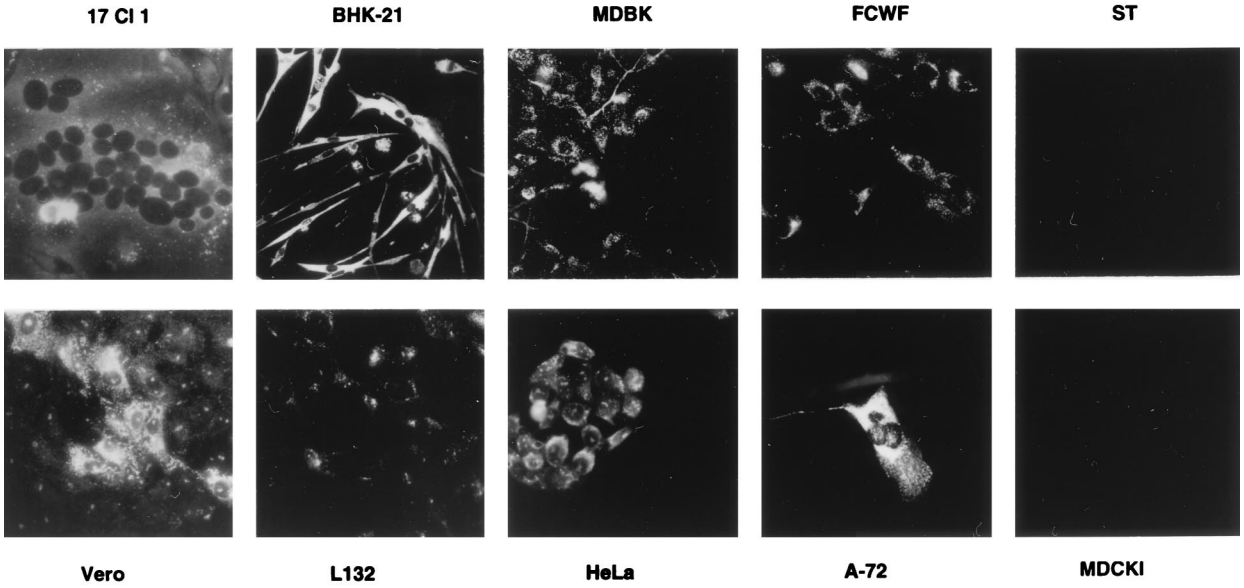

FIG. 9. Host range of MHV/BHK virus from persistently infected murine cells. Cell lines from different species were inoculated with MHV/BHK at an MOI of 3 to 5 PFU/cell. Murine 17 Cl 1 cells were fixed at 10 h p.i.; simian (Vero) and human (L132) cells were fixed at 17 h p.i.; and hamster (BHK), bovine (MDBK), feline (Fcwf), porcine (ST), human (HeLa), and canine (A-72 and MDCK I) cells were fixed at 24 h p.i. The presence of viral nucleocapsid in the cytoplasm of infected cells was detected by immunolabeling with mouse antinucleocapsid MAb and FITC-labeled anti-mouse IgG1. Magnification, ×200.

vivo, including morbilliviruses such as seal and horse morbilliviruses, arenaviruses such as Lassa fever virus and lymphocytic choriomeningitis viruses, arteriviruses such as porcine respiratory and reproductive syndrome virus, and retroviruses such as simian immunodeficiency virus type 1 and human immunodeficiency virus type 1 (1, 30). Persistent infection of human cells with measles virus, a morbillivirus, specifically down-regulates the expression of its CD46 receptor by a process that involves the first six amino acids in the cytoplasmic tail of the glycoprotein (23, 26). Down-regulation of the human immunodeficiency virus receptor CD4 also occurs in infected cells (28). For cells persistently infected with MHV, we do not yet know whether cells that express low levels of MHVR are selected by infection from a preexisting population of cells with low levels of receptor expression or result from virus-induced down-regulation of MHVR expression. In any case, we suggest that virus replication in cells expressing low MHVR levels and possibly with alternative MHV receptors of lower efficiency is important in selection of host range mutants of MHV from persistently infected cells. Adenovirus 2 mutants with altered receptor specificity are selected in persistently infected human cell lines that have markedly reduced binding affinity for the adenovirus 2 fiber antigen (18). Poliovirus variants have been selected for resistance to neutralization by soluble recombinant poliovirus receptor (9). Studies of these soluble receptor-resistant mutants have revealed sites on the virus capsid proteins that affect the affinity of receptor binding as well as sites that affect subsequent steps in virus uncoating. Analysis of the amino acid sequences of the S glycoproteins of many host range mutants of MHV-A59 and other MHV strains derived from persistent infection of murine cell cultures or from soluble receptor-resistant mutants may reveal sites of the S glycoprotein that are associated with receptor binding and subsequent receptor-dependent processes such as virus-induced membrane fusion that play important roles in the species specificity of coronavirus infection.

This work points out the possible biohazards of working with persistently infected cell cultures or with viruses that have mutations in their receptor-binding proteins, because either of these could yield viral host range mutants. It is not yet possible to predict the host range, tissue tropism, or virulence of variant viruses with altered receptor specificities, so care should be taken to prevent the spread of the variant viruses.

Data presented here shows that virus variants with altered receptor specificity and host range can be selected during persistent infections in vitro in which the expression of the virus receptor is low, possibly with the presence of alternative virus receptors of lower efficiency. When virus strains are found to have altered host range, it is important to review their passage history to determine if growth under conditions of limited receptor availability could have selected for the observed altered receptor specificity and/or host range variation. Several MHV strains have been previously reported to have extended host range, including MHV/JHM strains that can infect the central nervous system of young rats following intracerebral inoculation, infect primates, or utilize recombinant human CEA glycoproteins as receptors in vitro (4, 6, 8, 31, 42). It will be interesting to compare the sequences of the S glycoproteins of these viruses and other receptor specificity variants of MHV with the sequences of related MHV strains that infect only murine cells. The MHV spike protein binds to MHVR by its N-terminal S1 domain, including amino acids from 1 to 330 (39). The amino acids in the N-terminal domain of MHVR that recognize this virus spike glycoprotein have also been identified (34, 43). Amino acid sequences have been compared for the N-terminal domains of the murine CEA-related glycoproteins that can serve as alternative MHV receptors in murine cells (7, 8, 12, 32, 33, 47) and for Bgp-related glycoproteins of rats and humans which are potential receptors for MHV strains with extended host range (3, 16, 27). It should soon be possible to determine which amino acid changes in the receptor binding domain of the S1 protein are required to change receptor specificity. Analysis of receptor specificity mutants will provide valuable information about the evolution of viruses and the mechanisms of emergence of new viral diseases associated with the adaptation of virulent viruses to new hosts.

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