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# SELECTION IN PERSISTENTLY INFECTED MURINE CELLS OF AN MHV-A59 VARIANT WITH EXTENDED HOST RANGE

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## 1. ABSTRACT

Murine coronavirus MHV-A59 normally infects only murine cells *in vitro* and causes transmissible infection only in mice. In the 17 Cl 1 line of murine cells, the receptor for MHV-A59 is MHVR, a biliary glycoprotein in the carcinoembryonic antigen (CEA) family of glycoproteins. We found that virus released from the 600th passage of 17 Cl 1 cells persistently infected with MHV-A59 (MHV/pi600) replicated in hamster (BHK-21) cells. The virus was passaged and plaque-purified in BHK-21 cells, yielding the MHV/BHK strain. Because murine cells persistently infected with MHV-A59 express a markedly reduced level of MHVR (Sawicki, *et al.*, 1995), we tested whether virus with altered receptor interactions was selected in the persistently infected culture. Infection of 17 Cl 1 cells by MHV-A59 can be blocked by treating the cells with anti-MHVR MAb-CC1, while infection by MHV/BHK was only partially blocked by MAb-CC1. MHV/BHK virus was also more resistant than wild-type MHV-A59 to neutralization by purified, recombinant, soluble MHVR glycoprotein (sMHVR). Cells in the persistently infected culture may also express reduced levels of and have altered interactions with some of the Bgp-related glycoproteins that can serve as alternative receptors for MHV-A59. Unlike the parental MHV-A59 which only infects murine cells, MHV/BHK virus was able to infect cell lines derived from mice, hamsters, rats, cats, cows, monkeys and humans. However, MHV/BHK was not able to infect all mammalian species, because a pig (ST) cell line and a dog cell line (MDCK I) were not susceptible to infection. MHV/pi600 and MHV/BHK replicated in murine cells more slowly than

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MHV-A59 and formed smaller plaques. Thus, in the persistently infected murine cells which expressed a markedly reduced level of MHVR, virus variants were selected that have altered interactions with MHVR and an extended host range. *In vivo*, in mice infected with coronavirus, virus variants with altered receptor recognition and extended host range might be selected in tissues that have low levels of receptors. Depending upon the tissue in which such a virus variant was selected, it might be shed from the infected animal or eaten by a predator, thus presenting a possible means for initiating the transition of a variant virus into a new host as a model for an emerging virus disease.

## 2. INTRODUCTION

MHV is a murine coronavirus that naturally infects only mice, causing either inapparent infection or hepatitis, diarrhea, splenolysis or neurological disorders. The virus is transmitted via the respiratory and enteric routes, and spreads rapidly through mouse colonies (Compton, *et al.*, 1993). Though infection *in vivo* is usually limited to mice, a few exceptions have been reported. Following intracerebral inoculation, MHV/JHM can infect brain of rats and owl monkeys (Cabirac, *et al.*, 1994; Murray, *et al.*, 1992; Wege, *et al.*, 1982). MHV infection *in vitro* is also usually limited to mice. The principal receptor used by MHV-A59 to infect murine 17 Cl 1 cells, MHVR (also called $Bgp1^a$), is a biliary glycoprotein (Bgp) in the carcinoembryonic antigen (CEA) family (Dveksler, *et al.*, 1991). MHVR is expressed at high levels in mouse colon, small intestine, liver, trachea and smaller airways of the lung, kidney, and in lower levels on B lymphocytes and macrophages (Godfraind, *et al.*, 1995; Coutelier, *et al.*, 1994). Splicing of mRNA generates MHVR isoforms with either 2 or 4 Ig-like domains and either long or short cytoplasmic tails. Several isoforms can be co-expressed in murine cells. SJL/J mice are highly resistant to MHV-A59 infection. DNA sequence encoding MHVR is absent in SJL/J mice, but the genome encodes the $Bgp1^b$ (mmCGM2) glycoprotein in place of MHVR (Dveksler, *et al.*, 1993a). In tissue culture, $Bgp1^b$ and some additional murine Bgp-related glycoproteins can serve as MHV-A59 receptors if the recombinant proteins are expressed at high levels in cells of an MHV-A59 resistant species such as BHK or COS-7 cells. These receptors are Bgp2, which is a biliary glycoprotein with 2 Ig-like domains, and bCEA, a pregnancy specific glycoprotein (PSG) that is a soluble protein containing 3 Ig-like domains and lacking a membrane anchor (Chen, *et al.*, 1997; Chen, *et al.*, 1995; Nedellec, *et al.*, 1994).

Species specificity of MHV-A59 is determined, in part, by the virus-receptor interaction. MHV-A59 does not infect the BHK-21 line of hamster cells (called BHK below), but BHK cells transfected with cDNA encoding MHVR are susceptible to infection by MHV-A59. Anti-MHVR MAb-CC1 binds to the N-terminal domain of MHVR that is present in both the 4-domain and 2-domain isoforms of MHVR, and blocks MHV-A59 infection of murine cells and MHVR-transfected BHK cells (Dveksler, *et al.*, 1993b). The level of expression of MHVR is markedly reduced in murine 17 Cl 1 cells persistently infected with MHV-A59 and the cells are resistant to superinfection with wild type MHV-A59 (Sawicki, *et al.*, 1995). As summarized below, we found that virus from the persistently infected culture utilized MHVR in a different manner than MHV-A59 and may also utilize an additional receptor on persistently infected 17 Cl 1 cells (Schickli, *et al.* submitted). We also found that the virus from the persistently infected culture was able to infect cells of several non-murine species. We suggest that persistent infection of murine 17 Cl 1 cells with MHV-A59 causes a marked reduction in the expression of MHVR, which in turn selects for virus with altered interactions with MHVR and an extended host range.

## 3. RESULTS AND DISCUSSION

Cells from the 17 Cl 1 line of spontaneously transformed BALB/c mouse fibroblasts were inoculated with MHV-A59 at a high multiplicity of infection (100 PFU/cell) and then the cells were serially passaged (Sawicki, *et al.*, 1995). At passage 600, virus (MHV/pi600) from the supernatant medium was plaque-purified on 17 Cl 1 cells. Unlike the species-specific MHV-A59, MHV/pi600 could infect hamster (BHK) cells. This virus was serially passaged 12 times in BHK cells, then plaque-purified twice on BHK cells to yield MHV/BHK virus. Both MHV-A59 and MHV/BHK caused fusion of 17 Cl 1 cells, though fusion induced by MHV/BHK was less extensive (Figure 1). The plaques produced on mouse 17 Cl 1 cells by MHV/BHK were considerably smaller than those of MHV-A59 (0.5–1mm and 4–5mm, respectively).

To determine if MHV/BHK infects 17 Cl 1 cells by interacting with MHVR, we used anti-MHVR MAb-CC1 to block virus binding to MHVR. The cells were incubated for one hour at 37°C with supernatant medium from hybridomas producing either MAb-CC1 or a control MAb and then inoculated with either MHV-A59 or MHV/BHK at 3 PFU/cell. After three hours at 37°C, the inocula were removed and the cells were refed with medium containing either MAb-CC1 or control MAb. Virus released into the supernatant medium at 4, 9.5, and 21 hours after virus inoculation was titered in 17 Cl 1 cells using a $TCID_{50}$ assay.

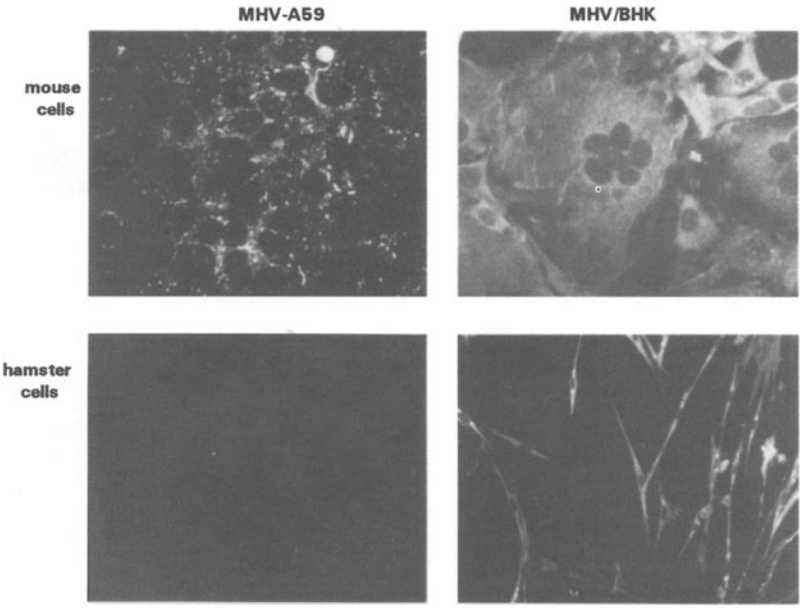


**Figure 1.** Susceptibility of murine (17 Cl 1) cells and hamster (BHK) cells to infection with MHV-A59 or MHV/BHK. Cells were inoculated with either MHV-A59 or MHV/BHK derived from persistently infected murine cells at 3 to 5 PFU/cell and fixed in acetone at 10 hr. after inoculation for mouse cells or 24 hours for hamster cells. Viral nucleocapsid protein was detected in the cytoplasm of infected cells by immunolabeling. MHV/BHK infected both mouse and hamster cell lines, while the parental MHV-A59 virus infected only mouse cells.

**Table 1.** Yields of MHV-A59 and MHV/BHK from murine 17 Cl 1 cell treated with anti-MHVR MAb-CC1. Virus yields of MHV-A59 or MHV/BHK at the indicated time points, in the presence of either anti-MHVR MAb-CC1 or a control MAb, were determined using a $TCID_{50}$ assay in 17 Cl 1 cells

| | MHV-A59 | | MHV/BHK | |
|---|---|---|---|---|
| Time of harvest** | Control MAb | MAb-CC1 | Control MAb | MAb-CC1 |
| 4.0 | 1.5* | 0 | 2.5 | 0 |
| 9.5 | 4.8 | 0 | 3.5 | 1.5 |
| 21.0 | 6.5 | 0 | 6.3 | 5.3 |

*$\log_{10}$ $TCID_{50}$
**Hours

While MAb-CC1 completely blocked MHV-A59 infection, it only partially inhibited infection of 17 Cl 1 cells by MHV/BHK (Table 1). This indicates that MHV/BHK can use MHVR as a receptor, but in a different manner from MHV-A59 and/or that MHV/BHK may also use an alternative receptor to initiate infection of 17 Cl 1 cells.

We also incubated MHV-A59 or MHV/BHK with varying concentrations of purified recombinant, soluble MHVR containing all four Ig-like domains (sMHVR) and then assayed for virus infectivity. The sMHVR neutralized MHV-A59 more than 100 fold more effectively on a micromolar basis than it neutralized MHV/BHK virus. Because MHV/BHK was neutralized by high concentrations of sMHVR , the MHV/BHK virus is apparently able to utilize MHVR, although much less effectively than MHV-A59.

Genomes of all MHV strains contain a gene encoding a hemagglutinin esterase (HE) glycoprotein that can bind to 9-O-acetylated neuraminic acid residues on membrane macromolecules (Vlasak, *et al.*, 1988). In wild type MHV-A59, although the HE gene is present in the viral genomic RNA, no HE protein is expressed due to 3 different mutations (Yokomori, *et al.*, 1991). We addressed the possibility that the mutant MHV/BHK virus had acquired the ability to express HE which would allow it to bind to 9-O-acetylated sialylic acid residues on host cells, possibly facilitating virus entry. 17 Cl 1 cells were inoculated with 10-fold serial dilutions of either MHV-DVIM which expresses HE (Gagneten, *et al.*, 1995; Sugiyama, *et al.*, 1986), wild type MHV-A59 which does not express HE, or MHV/BHK. After 1 hour at 37°C, the virus inocula were removed and gel overlay was applied. At 2 days (MHV-A59) or 3 days (MHV-DVIM and MHV/BHK) after inoculation

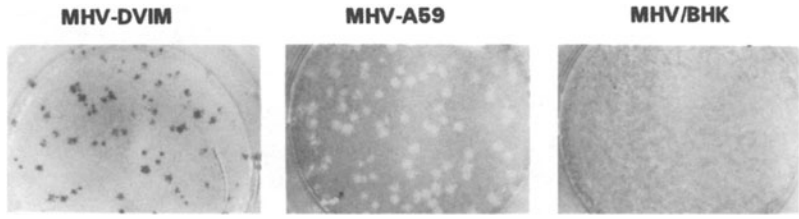


**Figure 2.** Acetyl esterase activity of hemagglutinin esterase (HE) glycoprotein in MHV plaques. Virus plaques on 17 Cl 1 cells were fixed 2 days after inoculation with MHV-A59 and 3 days for MHV-DVIM and MHV/BHK. The red product of the esterase activity was seen in plaques of MHV-DVIM, but not in plaques of MHV-A59 or MHV/BHK.

**Table 2.** Host range of MHV/BHK virus obtained from murine cells persistently infected with murine coronavirus MHV-A59

| | Susceptibility to infection with | |
|---|---|---|
| Cell lines | MHV-A59 | MHV/BHK |
| Mouse | + | + |
| Hamster | – | + |
| Rat | – | + |
| Cat | – | + |
| Cow | – | + |
| Human | – | + |
| Dog | – | +/– |
| Pig | – | + |

the gel was removed, and the cells were fixed in cold formalin/acetone and tested for esterase activity as described by Wagaman , *et al.* (1989) (Figure 2). Plaques of the positive control, MHV-DVIM, showed deep red staining on the infected cells, while plaques of MHV-A59, which served as a negative control, showed no esterase activity. Plaques of MHV/BHK also did not show esterase activity. Thus, the HE gene in this virus is not expressed and HE glycoprotein is absent from the envelope of MHV/BHK virions so it cannot bind to an alternative carbohydrate moiety as a receptor.

MHV/BHK developed the ability to infect hamster cells during persistent infection in mouse 17 Cl 1 cells without prior exposure to hamster cells. We investigated whether cell lines of species other than mouse and hamster were also susceptible to MHV/BHK infection. Infection by MHV/BHK was assayed by immunofluorescence. The cells were fixed in -20°C acetone at 10, 17, or 24 hours after inoculation, and nucleocapsid antigen was detected with anti-N MAb and FITC-labeled goat anti-mouse IgG as previously described (Dveksler, *et al.*, 1991). The results are summarized in Table 2. We found that rat (RIE), cat (Fcwf), cow (MDBK) and human (L-132 and HeLa) cell lines were susceptible to infection by MHV/BHK. In these cell lines, 5% to 100% of the cells were infected by 24 hours after virus inoculation. One dog cell line (A72) was only slightly susceptible to MHV/BHK infection, as <1% of the cells contained viral antigen at 24 hours after inoculation. The dog kidney (MDCK I) and the swine testis (ST) cell lines were completely resistant to MHV/BHK infection.

Infection of the non-murine cell lines by MHV/BHK did not induce extensive cell fusion. In hamster cells, <1% of the cells were binucleated by 24 hours after inoculation. No fusion was seen in any of the non-murine cell lines tested (Figure 1). However, MHV/BHK was able to induce fusion in murine 17 Cl 1 cells, though to a lesser extent and more slowly than MHV-A59. OBLV60, an acid-dependent variant of MHV-4 (JHM-MHV) was selected during persistent infection in OBL21a cells that are resistant to fusion (Gallagher, *et al.*, 1991). OBLV60 enters OBL21a and Sac- cells via the endocytic pathway. In contrast, MHV-4 virus grown in Sac- cells that are not resistant to fusion enters Sac- cells via both endocytic and non-endocytic pathways (Nash and Buchmeier, in press). Possibly, MHV/BHK infection of murine cells also can be initiated both by the endocytic pathway and by direct fusion at the plasma membranes, while infection of the non-murine cells may only occur via the endocytic pathway.

In addition to the differences in cell fusion activity between MHV-A59 and MHV/BHK, differences in the rates of viral RNA synthesis were observed. The rate of viral RNA synthesis was determined by measuring Actinomycin D-resistant RNA synthesis as described by Sawicki and Sawicki (1986) in 17 Cl 1 cells or BHK cells infected with either MHV-A59, MHV/pi600 or MHV/BHK (Schickli, *et al.*, submitted). In 17 Cl 1 cells infected with MHV-A59, viral RNA synthesis was maximal at 7 hours after inoculation, while in cells infected with either MHV/pi600 or MHV/BHK, the rate of viral RNA synthesis peaked at 10 hours and was 3-fold and 2-fold lower, respectively, than MHV-A59 (data not shown). In BHK cells, the synthesis of MHV/pi600 or MHV/BHK viral RNA was considerably slower than for the same viruses in murine 17 Cl 1 cells. The rate of MHV/BHK RNA synthesis at 24 hours after inoculation of BHK cells was roughly 3-fold less than that of 17 Cl 1 cells infected with MHV-A59 at 7 hours (data not shown). Perhaps MHV-A59 has adapted to maximize viral RNA synthesis in murine cells.

The MHV/BHK virus formed small plaques on both 17 Cl 1 and BHK cells, had altered interactions with MHVR, was less fusogenic in murine cells and had an extended host range compared to MHV-A59. MHV/BHK virus was selected in persistently infected murine cells that express low levels of MHVR (Sawicki, *et al.,* 1995). Alternative MHV receptors such as Bgp2 may also be expressed on these cells, so mutant virus that can utilize the alternative receptor(s) better than MHV-A59 may have a selective advantage in the persistently infected murine cell culture. Murine tissues differ markedly in their level of expression of MHVR (Godfraind, *et al.*, 1995). High levels of MHVR are found in respiratory and enteric epithelium, portals of entry and shedding of MHV, while other tissues such as spleen and brain express much less MHVR. *In vivo*, in MHV-infected murine tissues that express low levels of MHVR, mutant virus that has an extended host range may be selected as in the persistently infected murine cell culture. Mutant virus would probably not be shed effectively from these tissues, although it might play a role in the disease process within the infected mouse. If a mouse infected with such a host range virus variant were ingested by a predator or scavenger, the mutant virus might gain the opportunity to initiate infection in a new host species, possibly leading to the emergence of a new virus disease.

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