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Persistent Infection of Cultured Cells with Mouse Hepatitis Virus (MHV) Results from the Epigenetic Expression of the MHV Receptor

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The A59 strain of murine coronavirus mouse hepatitis virus (MHV) can cause persistent infection of 17C1-1 cells and other murine cell lines. Persistently infected cultures released large amounts of virus (10^7 to 10^8 PFU/ml) and were resistant to superinfection with MHV but not to infection with unrelated Semliki Forest and vesicular stomatitis viruses. The culture medium from persistently infected cultures did not contain a soluble inhibitor such as interferon that protected uninfected cells from infection by MHV or vesicular stomatitis virus. The persistent infection was cured if fewer than 100 cells were transferred during subculturing, and such cured cultures were susceptible to reinfection and the reestablishment of persistent infection. Cultures of 17C1-1 cells that had been newly cloned from single cells consisted of a mixture of MHV-resistant and -susceptible cells. 17C1-1/#97 cells, which were cured by subcloning after 97 passages of a persistently infected culture over a 1-year period, contained 5 to 10% of their population as susceptible cells, while 17C1-1/#402 cells, which were cured by subcloning after 402 passages over a 3-year period, had less than 1% susceptible cells. Susceptibility to infection correlated with the expression of MHV receptor glycoprotein (MHVR [Bgp1[a]]). Fluorescence-activated cell sorter analysis with antibody to MHVR showed that 17C1-1/#97 cells contained a small fraction of MHVR-expressing cells. These MHVR-expressing cells were selectively eliminated within 24 h after challenge with MHV-A59, and pretreatment of 17C1-1/#97 cells with monoclonal antibody CC1, which binds to the N-terminal domain of MHVR, blocked infection. We conclude that the subpopulation of MHVR-expressing cells were infected and killed in acutely or persistently infected cultures, while the subpopulation of MHVR-nonexpressing cells survived and proliferated. The subpopulation of MHVR-negative cells produced a small proportion of progeny cells that expressed MHVR and became infected, thereby maintaining the persistent infection as a steady-state carrier culture. Thus, in 17C1-1 cell cultures, the unstable or epigenetic expression of MHVR permitted the establishment of a persistent, chronic infection.

Mouse hepatitis virus (MHV) is a member of the coronavirus family of enveloped, positive-strand RNA viruses and causes both lytic and persistent infections in vivo and in vitro. Inbred mouse strains differ in their susceptibility or resistance to infection (reviewed in reference 19). One factor correlated with MHV susceptibility of mouse strains is the expression by target tissues of a 110-kDa MHV receptor glycoprotein (MHVR) and its isoforms (2, 31). Isoforms of MHVR result from alternative splicing and have two or four immunoglobulin (Ig)-like extracellular domains and long or short intracytoplasmic tails (1, 3, 32, 34). MHVR has been defined as a biliary glycoprotein (Bgp1[a]) in the murine carcinoembryonic antigen superfamily (9, 18, 31). It is found in a wide range of tissues, including principally the liver and intestinal epithelium (2–4, 31, 34). The level of expression of MHVR isoforms differs markedly among cultured mouse cells (13a).

Most coronaviruses cause acute and self-limited infections in a single host species or in a few closely related species. However, persistent infections can be established in immunosuppressed or neonatal animals (6, 19, 25, 30). MHV infection of many different cultured murine cell lines leads to the efficient development of persistent infections in vitro. The persistently infected cultures can produce defective noninterfering viruses, temperature-sensitive or cold-sensitive viruses, non-temperature-sensitive viruses with wild-type phenotypes, and small-plaque variants (10–14, 16, 17, 20, 21, 23, 25, 26, 29). Many of the latter are defective in cell fusion (8, 13, 21, 29) and altered in virulence (10). Many of the small-plaque variants possess a mutation in the 180-kDa viral spike (S) glycoprotein that changes a His residue within the highly basic amino acid sequence required for protease cleavage into its 90-kDa subunits (10). Persistently infected cultures are resistant to superinfection with homologous viruses but not with heterologous viruses such as vesicular stomatitis virus (VSV) (13). Numerous mechanisms have been suggested for the establishment and/or maintenance of persistence in vitro that include roles for mutant or interfering viruses (15, 26), for latently infected cell populations (25), and for a host cell-regulated subpopulation of cytopathically infected cells in a carrier culture (13, 16, 17). In this report, we show that the establishment of a persistent infection in cultured cells results from the epigenetic expression of MHVR.

(Some of the results were presented at the 1994 meeting of the American Society for Virology.)

MATERIALS AND METHODS

Cells and viruses. The spontaneously transformed mouse BALB/c 3T3 cells, 17C1-1 cells (28), and the A59 strain of MHV were kindly provided by L. Sturman. The 17C1-1 cells used in this study were cloned from a single cell and grown at 37°C and 7.5% CO_2 in Dulbecco's modified Eagle medium containing 6% fetal bovine serum (FBS), 5% tryptose phosphate broth (TPB), 500 U of penicillin per ml, and 100 U of streptomycin per ml (neutral pH growth medium). The cultures were routinely passaged every 3 days at a dilution of 1:60. The cells were detached from the monolayer by incubation in Hanks' balanced salt

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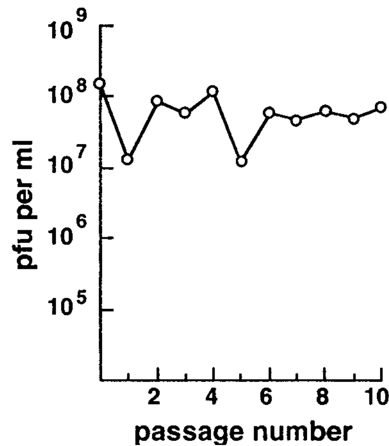

FIG. 1. Virus yields of 17C1-1 cells persistently infected with MHV-A59. Monolayers of 17C1-1 cells were inoculated at an MOI of 100 PFU per cell with MHV-A59 and maintained in low-pH growth medium at 37°C and 10% CO_2. The cultures were refed with neutral-pH medium at 24 and 48 h p.i. and passaged undiluted after 72 h p.i. Thereafter, the culture was passaged every 3 days, and the amount of virus released into the medium after 3 days at each passage level was assayed on 17C1-1 cells.

solution that lacked Mg^{2+} and Ca^{2+} and contained 0.25% trypsin (1:250) and 1 mM EDTA. Cultures persistently infected with MHV-A59 were derived from 17C1-1 cells as described in the legend to Fig. 1. The passage history is indicated by the letter "p" followed by the passage number (i.e., PI#3p203 indicates cells at the 203rd passage after their initial infection). PI#1 was the first independently established persistently infected culture, and PI#3 was the third. 17C1-1/#97 (#97) indicates cells derived by single-cell cloning from PI#3p97 cells; subsequently, they have been grown continuously for over 100 passages and are a stable cell line. Similarly, 17C1-1/#402 (#402) cells were subcloned from PI#3p402 cells. After about 10 passages as described in the legend to Fig. 1, the PI#3 cells, as well as the #97 and #402 cells, were routinely passaged every 3 days at a dilution of 1:15 or 1:30 in order to obtain a confluent monolayer by 3 days.

The alphavirus Semliki Forest virus (SFV) was grown as described previously (22). The Indiana strain of vesicular stomatitis virus (VSV) was a gift from R. Herman and was propagated in 17C1-1 cells. MHV-sp1, MHV-pi26, MHV-pi203, and MHV-pi263 are viruses isolated from PI#1p40 (21), PI#3p26, PI#3p203, and PI#3p263, respectively. They were doubly plaque purified before stocks were prepared. MHV was propagated in 17C1-1 cells, and high-titer stocks were prepared by infecting a confluent monolayer in 150-mm-diameter petri dishes with MHV diluted to a multiplicity of infection (MOI) of 0.01 to 0.1 PFU per cell in Dulbecco's phosphate-buffered saline (PBS) containing 0.2% bovine serum albumin, 0.01 volume of 2 M HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [pH 6.6]), 50 μg of DEAE-dextran (Sigma Chemical Co., St. Louis, Mo.) per ml, 500 U of penicillin per ml, and 100 U of streptomycin per ml. DEAE-dextran in the inoculation medium enhanced the infectivity of MHV-A59 and MHV-sp1 but not MHV-pi26, MHV-pi203, and MHV-pi263 and caused a fivefold increase in the virus titer (PFU per milliliter). After inoculation, the virus was adsorbed to the cells at room temperature on a mechanical rocking platform for 1 h. After the adsorption period, the virus inoculum was removed, the cell monolayer was rinsed once with PBS, 29 ml of low-pH Dulbecco's modified Eagle medium (0.75 g instead of 3.7 g of sodium bicarbonate per liter, 8.45 g instead of 6.4 g of sodium chloride per liter, and 10 ml of 2 M HEPES [pH 6.6] per liter) containing 12% FBS, 5% tryptose phosphate broth, 500 U of penicillin per ml, and 100 U of streptomycin per ml (low-pH growth medium) was added, and the cultures were incubated overnight at 37°C and 10% CO_2. Under these conditions, the pH of the culture medium was at 6.6 to 6.8, which prevented or delayed cell fusion and resulted in virus titers of (2 to 4) $\times$ 10^9 PFU/ml or about 10-fold higher than is achieved with neutral-pH growth medium (22). Virus titers were determined by plaque assay on 17C1-1 cells in 60-mm-diameter petri dishes. After inoculation and adsorption, Dulbecco's modified Eagle medium containing 6% FBS, 0.11% Gelrite (Kelco, San Diego, Calif.), 500 U of penicillin per ml, and 100 U of streptomycin per ml (Gelrite overlay medium) was added. The cells were incubated at 37°C and 7.5% CO_2 for 2 days until plaques 4 to 6 mm in diameter appeared, at which time, the Gelrite overlay medium was decanted and the cell monolayer was fixed with methanol and stained with 0.2% azure II and 0.2% toluidine blue in 1% boric acid to visualize the plaques.

Antisera. Monoclonal antibody (MAb) CC1 is directed against the N-terminal domain of the MHVR ($Bgp1^a$) (5, 18) that is expressed by MHV-susceptible BALB/c, C57B/6, and Swiss mice. MAb CC1 recognizes the glycoprotein produced by all splice variants of $Bgp1^a$ but does not recognize $Bgp1^b$ glycoprotein that is expressed in SJL/J mice (3) or the Bgp2-related glycoprotein that is expressed in both BALB/c and SJL/J mice (18). MAb CC1 blocks MHV-A59 infection of 17C1-1 cells and L2 murine fibroblasts (24). Goat polyclonal anti-S antiserum was raised by immunization with viral S glycoprotein purified from MHV-A59 virions (24, 32). A MAb specific for the MHV nucleocapsid (N) protein (anti-N) was a gift from J. Leibowitz. Rabbit anti-goat or rabbit anti-mouse antibodies conjugated with rhodamine or fluorescein isothiocyanate (FITC) were obtained from Sigma Chemical Co.

Immunofluorescence. Cells on coverslips were fixed at −20°C with acetone, air dried, and stored at −20°C. The fixed cells were rehydrated in PBS containing 1% FBS and incubated with diluted goat anti-S antibody for 60 to 90 min at 37°C in a moist chamber followed by rhodamine- or FITC-conjugated rabbit anti-goat Ig. Cells were also labeled with anti-N MAb followed by fluorescein-conjugated rabbit-anti-mouse Ig. The cells were counterstained with Evans blue or propidium iodide.

Detection of MHVR. The expression of MHVR was assessed by fluorescence-activated cell sorter (FACS) analysis. The cells were harvested by washing the cell monolayers with PBS-EDTA followed by mild trypsinization. All subsequent procedures were at 4°C. The cells were washed with cell culture medium, counted, and transferred to labeling buffer (Hank's buffered salt solution containing 3% FBS). MAb CC1 or a control MAb of the same IgG1 isotype but directed against an irrelevant antigen was added to one million cells in 100 μl of buffer to a final concentration of 40 μg of antibody per ml. After 30 min of incubation, the cells were washed twice with 2 ml of labeling buffer and incubated for 30 min with phycoerythrin-coupled goat anti-mouse IgG1 antiserum (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). The cells were washed twice, resuspended in a final volume of 0.5 ml, and analyzed with an EPICS Elite cell sorter (Coulter Electronics, Hialeah, Fla.).

Receptor blockade. Monolayers of 17C1-1 cells and #97 cells on coverslips were pretreated with a 1:2 dilution of supernatant medium from the hybridoma culture of anti-MHVR MAb CC1 for 1 h and then inoculated with MHV-A59 at an MOI of 5 PFU per cell in the presence of MAb CC1 and incubated at 37°C for 1 h. The inoculum was removed, and cultures were refed in medium without antibody and then incubated until 7 to 9 h postinfection, when the cells were fixed in cold acetone and processed for indirect immunofluorescence with mouse anti-S antiserum as the primary antibody.

RESULTS

Establishment and maintenance of persistently infected 17C1-1 cells. Figure 1 illustrates the yield of virus produced during the first 10 passages of 17C1-1 cells persistently infected with MHV. Confluent monolayers of 17C1-1 cells were inoculated with MHV-A59 at an MOI of 100 PFU per cell. After the 1-h adsorption period, the viral inoculum was removed and the cells were refed with low-pH growth medium. The cells were refed daily, and by the third day more than 95% of the cells had detached from the monolayer. If neutral-pH growth medium had been used instead of low-pH growth medium, the monolayer formed a giant syncytium that pealed off by 18 h postinfection (p.i.), leaving behind a small number of single cells that had not fused into the syncytium. On the third day postinoculation, the surviving cells were passaged undiluted and transferred to a new dish with neutral-pH growth medium. Thereafter, every 3 days, the growth medium was removed for virus titration and the cells were passaged, initially at a 1:2 dilution and then later up to a 1:15 or 1:30 dilution as needed to prevent overcrowding. The yield of virus in the culture medium determined on the day the cells were subcultured remained in the range of 10^8 PFU/ml (10 to 100 PFU per cell) throughout the first 10 passages. During the first 50 passages, if fewer then 10^4 cells were transferred into a 25-cm^2 flask, then a crisis would develop that consisted of a wave of cell fusion followed by cytolysis. This suggested that cells susceptible to infection accumulated and became acutely infected. The persistently infected culture was maintained by transfer of a sufficient number of cells to allow the monolayer to become confluent within 3 days.

One 17C1-1 culture persistently infected with MHV-A59, referred to as PI#3 cells, was carried for more than 400 passages over a 3-year period. The yield of released virus gradually declined from 10^7 to 10^8 PFU/ml to 10^6 to 10^7 PFU/ml over 400 passages (data not shown). The virus produced during the

first 10 passages had the same large-plaque morphology as the parental MHV-A59 that was used to establish the persistent infection. Between passages 10 and 50, the released virus produced plaques of various sizes. One variant, called MHV-sp1, obtained after 40 cell passages of PI#1 cells over a 6-month period, lacked the ability to cause cell fusion (21). After 100 passages of the PI#3 culture, all of the virus produced by the persistently infected culture exhibited a small-plaque morphology on uninfected 17C1-1 cells and showed delayed cell fusion compared with MHV-A59. The small-plaque viruses isolated between 100 and 400 passages were lytic and grew efficiently at 37°C.

Characterization of virus-cell interactions in persistently infected cultures. Beginning at passage number 97, we determined the fraction of cells that expressed the viral S glycoprotein or the nucleocapsid (N) protein. One day after subculturing PI#3p97 cells, 12% of the cells expressed the S glycoprotein and N protein, and by the second day after subculture, only 3% of the cells expressed these viral proteins. All of the cells that expressed the S glycoprotein also expressed the N protein. Little cell fusion was observed in the persistently infected culture, although the virus produced was capable of causing cell fusion, albeit delayed, when assayed on uninfected 17C1-1 monolayers. Cells expressing viral proteins appeared to be in various stages of the infectious cycle. Except for the fact that they expressed viral proteins, some had the same morphology as the uninfected cells, while other cells had lysed and left behind remnants containing viral antigens. In periodic analyses at intervals during the first 100 passages, 10 to 15% of the PI#3 cells 1 day after passage were found to express the S glycoprotein.

After passage 100, the persistently infected cultures produced only virus with a small-plaque morphology of <2 mm in diameter after 2 days at 37°C; by 4 days, the plaques were approximately the size of those produced by MHV-A59 at 2 days. Therefore, we could determine if the persistently infected cultures were susceptible to superinfection with MHV-A59, which produced plaques >5 mm in diameter after 2 days at 37°C. When persistently infected cultures were washed extensively and inoculated with MHV-A59 at an MOI of greater than 100 PFU per cell, they could not be superinfected (data not shown). These superinfected cultures produced only virus with a small-plaque morphology and in amounts equal to that produced by a parallel persistently infected culture that was not superinfected with parental MHV-A59. To determine if a soluble inhibitory factor such as interferon was produced by persistently infected cultures and protected the cells from superinfection, the persistently infected cultures were used for plaque assay of unrelated viruses VSV and SFV. The titers of VSV and SFV obtained with the persistently infected monolayers were nearly the same as those obtained with uninfected 17C1-1 cells, and the persistently infected cultures failed to form plaques when infected with MHV-A59 (Table 1). We determined if there was a inhibitor, such as a soluble form of the viral S glycoprotein or of the MHVR, in the culture medium of the persistently infected cells that would specifically protect 17C1-1 cells from infection with MHV-A59. Culture medium from a persistently infected culture that had been subcultured 3 days before was subjected to ultracentrifugation to reduce the titer of PI virus to less than 10 PFU/ml. 17C1-1 cells were refed with this medium for 30 h before they were used for plaque assay of MHV-A59 or VSV. The titers of both viruses, which produced large clear plaques by 2 days and hence could be clearly distinguished from any residual PI virus, were the same on the treated cells as on the untreated cells (Table 1). Thus, persistently infected cultures were resistant to infection by the homologous virus MHV-A59, but not by the heterologous viruses VSV and SFV, and the persistently infected cells did not produce a soluble inhibitor that prevented infection of 17C1-1 cells by homologous or heterologous viruses.

TABLE 1. Persistently infected cultures were susceptible to infection by heterologous but not homologous virus and did not secrete an inhibitor of MHV-A59 infection

Infection	Titer (PFU/ml)			
	Expt 1[a]		Expt 2[b]	
	17C1-1	PI#3p203	Untreated	Treated
Superinfecting virus				
VSV	1.3×10^9	5.4×10^9		
SFV	2.2×10^9	2.6×10^9		
MHV-A59	2.0×10^8	<10		
Virus				
MHV			2.7×10^8	2.1×10^8
VSV			1.1×10^9	1.3×10^9

[a] 17C1-1 or PI#3p203 cells were passaged to 60-mm-diameter petri dishes the day before they were used to determine the titer by plaque assay with SFV, VSV, and MHV-A59. The titer is the average of three dishes.

[b] Medium from PI#3p88 cells was collected, pooled, and centrifuged at 15,000 × g for 10 min and then centrifuged twice at 150,000 × g for 3 h. The top half of the supernatant was collected, sterilized by filtration through 0.22-μm-pore-size filters, and added to 17C1-1 monolayers in 60-mm-diameter petri dishes. The cells were incubated at 37°C for 30 h and then used to determine the titer of VSV or MHV. The untreated cells were a duplicate set of cultures fed with fresh growth medium. The titer is the average of three dishes.

Subcloning of 17C1-1 cells and of cells from persistently infected cultures. If fewer than 100 cells were subcultured, then the persistent infection ended and the resulting cultures no longer produced virus and became susceptible to infection with MHV-A59 virus. In order to maintain the persistent infection, more than 50 cells had to be transferred during the first 10 passages of a newly established persistently infected culture, and after 100 passages, at least 1,000 cells needed to be transferred during subculturing. Therefore, we were unable to obtain persistently infected cells that were derived by cloning

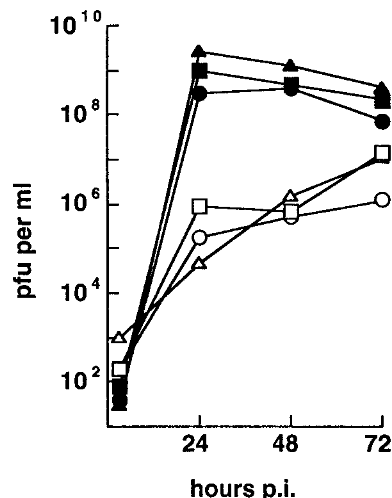

FIG. 2. Levels of virus produced by 17C1-1 cells (solid symbols) and #97 cells (open symbols) challenged with 20 PFU of MHV-A59 (circles), MHV-pi26 (squares), or MHV-pi203 (triangles) per ml. The yield of virus was determined with 17C1-1 cells as described in Materials and Methods.

TABLE 2. Clones of 17C1-1 cells and #97 cells were mixtures of MHV-susceptible and MHV-resistant cells

Cells	Value at MOI (PFU/cell) of[a]:			
	200		25	
	No. of cells counted	% of cells expressing S glycoprotein	No. of cells counted	% of cells expressing S glycoprotein
17C1-1/A	800	97.5	1,093	96.2
17C1-1/B	985	84.2	943	81.5
#97	1,913	2.2	1,649	1.7

[a] 17C1-1/A and 17C1-1/B subclones of 17C1-1 cells and cured #97 cells, which had been subcloned from persistently infected cultures, were infected with MHV-A59 at either an MOI of 200 or 25 PFU per cell and monitored by immunohistochemistry for expression of the MHV S glycoprotein at 9 h p.i. as described in Materials and Methods.

from a single cell. All subclones derived from single cells were susceptible to infection with wild-type MHV-A59 (Fig. 2). The #97 cells were obtained by cloning from a single cell after 97 passages of the PI#3 culture and did not produce any virus. When they were inoculated with wild-type MHV-A59 or variants of MHV-A59 that were obtained from persistently infected cultures after 26 passages (MHV-pi26) or after 203 passages (MHV-pi203), they produced about 10- to 100-fold less virus than did 17C1-1 cells (Fig. 2). Therefore, cells cured of persistent infection were susceptible to MHV-A59 but produced less virus than 17C1-1 cells and showed minimal cytopathic effects. On the basis of observations that the resistance to homologous infection disappeared when the cells were cured of persistent MHV infection, that less than 10 to 15% of the cells in the persistently infected cultures were expressing viral S glycoprotein and N protein at any one time, and that the persistent infection was not maintained if fewer than 100 cells of the persistently infected culture were transferred during subculturing, we concluded the majority of cells in a persistently infected culture were uninfected.

We determined if all or only a few #97 cells were susceptible to infection with MHV-A59 and if 17C1-1 cells comprised a mixed population of susceptible and resistant cells. Table 2 shows an analysis determining virus susceptibility of two subclones, 17C1-1/A and 17C1-1/B, that had been subcloned from our laboratory stock of 17C1-1 cells within 2 weeks before plating on the coverslips and our laboratory stock of #97 cells. The 17C1-1/A subclone formed a round colony composed of rounded cells. The 17C1-1/B subclone formed an angular colony composed of spindle-shaped cells. These subclones of 17C1-1 cells and the #97 cells were inoculated with MHV-A59 at MOIs of 200 and of 25 PFU per cell, and the fraction of cells expressing the S glycoprotein was determined. The 17C1-1/A subclone was similar to our laboratory stock of 17C1-1 cells, in that greater than 95% of the cells expressed the viral S glycoprotein by 9 h p.i. when infected at MOIs of 200 and 25 PFU per cell. In contrast, less than 85% of the 17C1-1/B cells and less than 5% of the #97 cells expressed the viral S glycoprotein by 9 h p.i. at either MOI (Table 2). Because increasing the MOI from 25 to 200 PFU per cell did not greatly affect the percentage of cells that became infected, we concluded that certain cells in the culture were fully resistant to virus infection. If the affinity of the receptor for the virus was lower in the #97 cells, increasing the MOI should have caused a greater percentage of the cells to become infected. In fact, the minimum dose of virus, e.g., 3 to 10 PFU per cell, required to infect all of the cells in a culture caused the maximum percentage of the cells to become infected (data not shown).

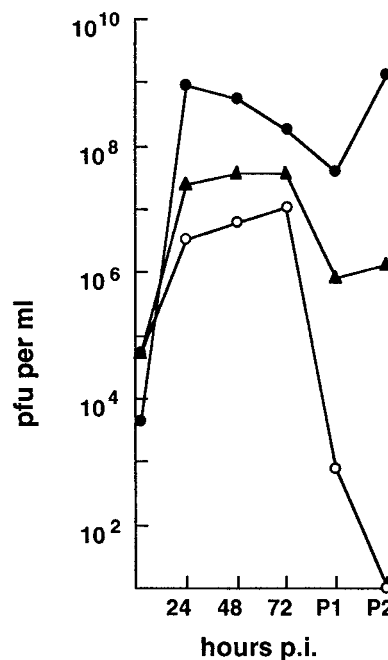

FIG. 3. Resistant populations of cells are selected with increased passage of the persistently infected cultures. Uninfected 17C1-1 cells (solid circles) and two cell lines cloned at different passages from the PI#3 cells were analyzed for their ability to be infected with MHV-A59. The #97 cells (solid triangles) were cloned after about 97 passages of the PI#3 cells, and the #402 cells (open circles) were cloned after 402 passages of the same PI#3 culture. Infection was with an MOI of 100 of wild-type MHV-A59, after which the cells were rinsed and refed at 1 h p.i. and again at 2 h p.i. and then maintained at 37°C. The medium was harvested from replicate sets of dishes at 3 h p.i. (this represents the virus remaining from the infecting inoculum) and at the times indicated. After 3 days, the cells were passaged. For P1, 17C1-1 cells were passaged undiluted, #97 and #402 cells were diluted 1:15, and the medium was harvested after 3 days. For P2, the cells were passaged a second time at the same dilution as P1 and the medium was harvested after 3 days. The titer (PFU per milliliter) was determined with 17C1-1 cells.

We determined if cured cells, e.g., #97 and #402 cells, would establish persistently infected cultures if inoculated with wild-type MHV-A59. On the basis of data shown in Fig. 3, #97 cells established a persistent infection, whereas #402 cells did not. After infection at an MOI of 100 PFU per cell, the culture of #402 cells produced about 10-fold less virus than did the culture of #97 cells and 100 to 1,000-fold less virus than did the culture of 17C1-1 cells. Upon passage, persistently infected cultures were obtained from both the infected 17C1-1 and #97 cells but not from infected #402 cells (Fig. 3). Greater than 95% of 17C1-1 and about 5% of #97 cells but less than 1% of the #402 cells produced the S glycoprotein after virus inoculation (data not shown). Thus, a large percentage of cured cells, which had been derived from persistently infected cultures, failed to become infected when challenged by high doses of virus. The resistant #97 and #402 cells that were susceptible to infection appeared to produce approximately the same amount of virus per infected cell as did the susceptible 17C1-1 cells. When the fraction of cells in a culture that are susceptible to infection with MHV-A59 drops below a certain level, as it appeared to do in #402 cells, the persistent infection was difficult to establish.

To determine if the susceptibility or resistance to MHV-A59 infection was genetically determined, we studied colonies derived from single 17C1-1 cells or #97 cells. About 10^3 cells of each type were added to 35-mm-diameter petri dishes that contained 22 by 22-mm-diameter coverslips, and the dishes

were incubated for 5 days until discrete colonies, each derived from a single cell, had formed. The dishes were then inoculated with $>10^9$ PFU (MOI of greater than 10^3 PFU per cell). Infection was monitored by determining the fraction of cells expressing the viral S glycoprotein (Fig. 4). At 9 h p.i., nearly all of the colonies of 17C1-1 cells had more than 95% of their cells expressing the S glycoprotein (Fig. 4A). Less than 5% of the 17C1-1 colonies had only 25 to 75% of their cells expressing the S glycoprotein (Fig. 4B and C). Significantly, we never observed a colony of 17C1-1 cells that was fully resistant to infection, i.e., that contained all uninfected cells. In contrast, colonies derived from #97 cells varied extensively in susceptibility to MHV-A59. About half of the colonies from #97 cells contained no infected cells, and the other half showed one to several infected cells (Fig. 4D; see also Fig. 7 below), but no colony had all or nearly all of the cells infected. Because we have been unable to clone from #97 cells or from #402 cells, a culture that was fully resistant to MHV-A59, we believe that eventually all of the colonies of #97 cells would have shown at least one cell that became infected with MHV-A59 if we would have allowed the cells to proliferate for longer than 5 days. Thus, cured cells derived from persistently infected cultures were much more resistant to infection than were 17C1-1 cells, but there was considerable variability in the fraction of cells in each colony that were susceptible to the virus.

Do cells that are resistant to MHV fail to express MHVR? We used FACS analysis with anti-MHVR MAb CC1 (24) to determine directly the relative levels of MHVR expression by the susceptible 17C1-1 cells, the resistant #97 cells, and infected #97 cells. More than 95% of the 17C1-1 cells expressed MHVR, but only a small proportion of #97 cells expressed MHVR (Fig. 5). Infection of #97 cells with MHV-A59 led to the selective elimination of the population of MHVR-expressing cells (Fig. 5). Infection of #97 cells with MHV-A59 was blocked by pretreatment of the cells with anti-MHVR MAb CC1 (Fig. 6). Therefore, the susceptible cells in the #97 culture expressed the same MHVR ($Bgp1^a$) as the parental 17C1-1 cells and utilized it as the functional receptor. However, the level of expression of MHVR on the susceptible cells in the #97 culture was very low, while resistant cells in the #97 culture did not express detectable MHVR. Infection of L2 cells, which are a derivative of L929 cells and express high levels of MHVR, resulted in the elimination of the MHVR-expressing cells and the selection of MHVR-nonexpressing cells by the protocol described in the legend to Fig. 1 (13a). These results, taken together with the results that used MHV infection to detect the MHVR, argue that MHVR expression was modulated in some unknown manner and that even though the cells in a culture or a clone were genetically identical, they were phenotypically mixed relative to the expression of MHVR. To further test the hypothesis that infection eliminated the MHVR-expressing cells and allowed the nonexpressing cells to survive and proliferate and that MHVR-expressing cells were produced from the nonexpressing cells, we infected #402 cells with MHV-A59 or MHV-pi263. As illustrated in Fig. 7, MHV-pi263 readily established a persistent infection of #402 cells but MHV-A59 did not (also see Fig. 3), although the same amount of each virus was produced, which was consistent with our suggestion that both viruses infected the same proportion of #402 cells. Both viruses produce $\sim 3 \times 10^9$ PFU/ml, or 100 more PFU of virus, on 17C1-1 cells compared with on #402 cells, and both viruses caused less than 1% of the #402 cells and greater than 95% of 17C1-1 cells to become infected when viral S glycoprotein expression was assessed. Upon passage and dilution of 1:30 of the infected cultures, the titer of MHV-A59 dropped to 10^3 to 10^4 PFU/ml, while the titer of MHV-pi263 remained between 10^6 and 10^7 PFU/ml. Upon the next passage (P2), MHV-A59 was undetectable. Reinfection of the P2 cells with MHV-A59 again produced a titer of 10^6 to 10^7 PFU/ml. We conclude that in cultures of #402 cells, like the #97 cells, the majority of the cells fail to express a sufficient level of MHVR to become infected, but these cells generate MHVR-expressing cells that become infected with MHV-A59 and die. Upon passage and dilution, the titer of MHV-A59 dropped below a level that appeared required to maintain the persistent infection and the MHVR-expressing cells that were generated did not become infected. When infected with a high titer of MHV-A59, these MHVR-expressing cells in P2 became infected and died. Therefore, infection eliminated the MHVR-expressing cells, but they reappeared within two passages and the culture was susceptible to reinfection by MHV-A59. We surmise that MHV-pi263, which appeared to cause cytolysis more slowly because it produces very small plaques that are cloudy even after 4 days at 37°C, allowed infected cells to survive and be transferred during passage. This permits more virus to be carried to the next passage. MHV-A59, on the other hand, killed cells more rapidly, which prevented the carriage of virus to the next passage. In #97 cells (Fig. 3), the number of MHVR cells that are being generated is high enough to maintain the persistent infection by MHV-A59. Therefore, it is the expression of the MHVR that determined persistent infection not the virus. Persistent infection appeared to select for cells that showed lower expression of MHVR, and this in turn appeared to select for virus that can survive in a culture that is populated with low numbers of MHVR-expressing cells.

DISCUSSION

Although 17C1-1 cells are highly susceptible to lytic infection with MHV-A59, persistent infection is readily established in these cells (13, 27, 28). To investigate the mechanism(s) for the establishment and maintenance of persistent infection, we assayed the expression of MHVR and the ability of cells in the uninfected and persistently infected cultures to produce viral structural proteins and infectious virions. Without prior exposure to virus, 17C1-1 cells and subclones derived from them were mixtures of virus-susceptible cells and virus-resistant cells. After infection, the virus-resistant cells survived the initial infection and proliferated. A small fraction of the progeny of these resistant cells was susceptible to infection by virus in the culture medium, and they served to maintain the persistent infection in the culture. In such persistently infected cultures, most cells were resistant to infection even when challenged with high levels of virus. Infection with MHV killed the susceptible cells that express the MHVR but not the resistant cells that did not express the MHVR detectable by FACS analysis.

In persistently infected cultures, the presence of uninfected cells was not due to too low a concentration of virus in the culture medium, as suggested by Mizzen et al. (17), but was due to cells that did not express the MHVR. There were two distinct cell populations in 17C1-1 cultures: the susceptible cells, which expressed the MHVR and underwent cell fusion and lysis, and the virus-resistant, MHVR-negative cells that were responsible for the subsequent establishment of persistent infection provided they eventually produced some MHVR-expressing, virus-susceptible cells. Similarly, #97 cells, which were mostly resistant to infection, occasionally produced cells that were susceptible to infection. Persistent infection of #97 cells was achieved with wild-type MHV-A59 as well as with MHV-pi26 or MHV-pi203 viruses obtained from persistently infected cultures. Therefore, some of the progeny of the

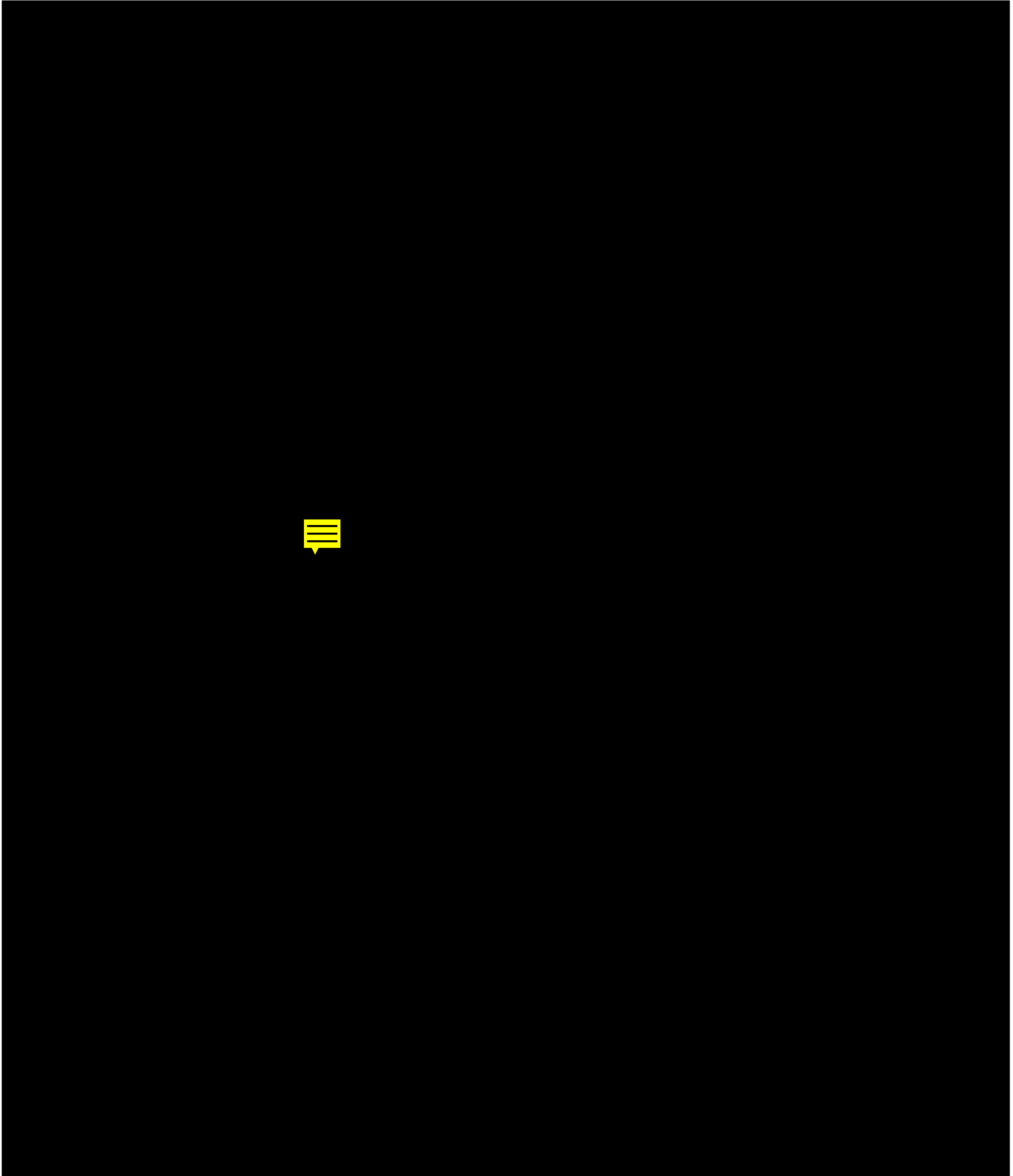

FIG. 4. Susceptibility and resistance of 17C1-1 cells (A to C) and #97 cells (D) to infection with MHV-A59. Infection was with an MOI of MHV-A59 of greater than 1,000 PFU per cell and was monitored at 9 h p.i. by immunofluorescence with anti-S glycoprotein antibodies and FITC-conjugated second antibody, as described in Materials and Methods. In panels A, B, and C, propidium iodide was used as a counterstain to visualize nuclei. Nuclei of cells expressing the S glycoprotein appear yellow because of the combination of the red propidium iodide and the green FITC; the nuclei of cells not expressing the S glycoprotein appear red. (A) Colony of 17C1-1 cells showing >95% of the progeny cells are susceptible to infection with MHV-A59. (B) Two 17C1-1 colonies show different levels of infectivity, one containing about 25% and the other containing about 75% of the total cells infected. (C) Colony of 17C1-1 cells showing only about 50% of the cells were susceptible to infection. (D) Colony of #97 cells containing a single infected cell in telophase. After staining the cells for the S glycoprotein as in panels A, B, and C, the cells were counterstained with Evans blue, which stains the cytoplasm red. When assessed at a later stage, only fluorescent remnants of such infected cells were found in #97 colonies, and the infection did not spread to any of the other cells in the colony (data not shown).

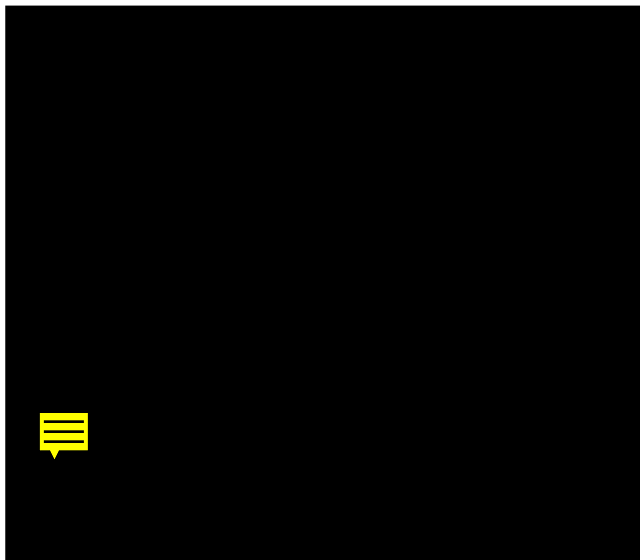

FIG. 4—*Continued.*

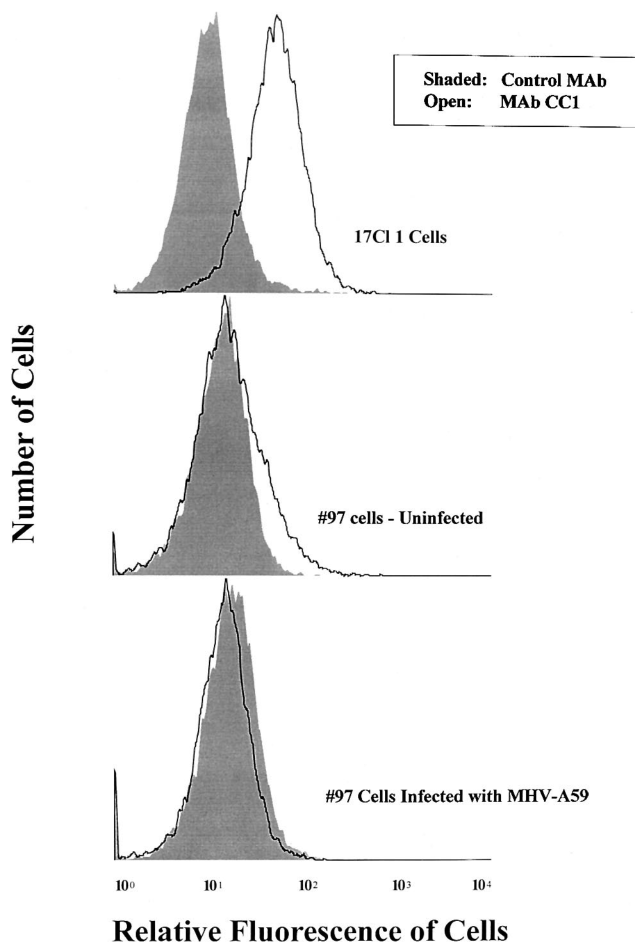

FIG. 5. Expression of the MHVR by cultures of 17C1-1 cells and uninfected and infected #97 cells. The level of MHVR was analyzed by FACS after staining with MAb CC1 as described in Materials and Methods. More than 95% of the 17C1-1 cells and less than 20% of the #97 cells expressed MHVR. After challenge of #97 cells with virus, no MHVR-expressing cells were found.

resistant cells must have been susceptible to infection, and, thus, susceptibility of 17C1-1 cells to MHV appears to be an epigenetic phenomenon. Once acquired, the resistant phenotype appears to be as stable as the susceptible phenotype: cultures of susceptible cells (17C1-1) and resistant cells (#97 cells) retained their phenotypes after being passaged continuously for more than 100 passages over a period of a year. Our results extend those of the earlier studies (13, 16, 17), which suggested that a small (1 to 10%) subpopulation of susceptible cells was essential for the maintenance of persistently infected carrier cultures. The reduced incidence of cell fusion in the persistently infected culture compared with that in the acutely infected cultures was attributed by other investigators to an inherent resistance of the LM-K cell membrane to MHV-induced fusion, so that the fusion resistance of cells was considered a major determinant of persistence (17). We observed that in 17C1-1 cells acutely infected at a high MOI with MHV-A59 and grown at pH 7.2 to 7.4, extensive cell fusion occurred and essentially the whole monolayer became fused into one giant multinucleated cell; however, the resistant cells remained as islands of single cells surrounded by the giant multinucleated cell and did not participate in cell fusion. On the basis of our results, we conclude that the membrane property missing from resistant cells (17) would be the MHVR. The mechanism responsible for the regulation of expression of MHVR is unknown.

The major cytopathology observed in acute MHV-A59 infection of 17C1-1 cells is cell fusion, which can be greatly reduced by culturing the cells in low-pH medium (22), when virus yield is increased by more than 10-fold. Nevertheless, MHV-infected cells succumbed to infection and died even when cell fusion was inhibited. This suggested that cell fusion inhibited virus production. After the initial acute infection, the persistently infected cultures did not show significant cytopathology or cell fusion. This was due to the low numbers of susceptible, infected cells rather than to an inability of the viruses that were produced by these cultures to cause cell fusion. It was only after more than 10 passages that fusion-deficient variants of MHV arose, as reported previously (13, 17). Although the persistently infected cultures were resistant to superinfection with MHV-A59, they were susceptible to infection with heterologous viruses, as found by others (11, 13, 23, 26). Thus, persistent infection with MHV-A59 selected cells expressing little or no MHVR but had little or no effect on the expression of receptors for unrelated viruses.

Similar to other reports (14, 17), persistent infection with MHV did not require mutant viruses. For at least the first 10 passages, the virus produced by the persistently infected cultures was not temperature sensitive or fusion deficient and gave the identical large-plaque phenotype of MHV-A59. With increased passage, virus variants that possessed altered cell fusion activity were produced, confirming previous reports (7, 8, 10, 13, 21). Sequencing of portions of the viral S glycoprotein gene of MHV-sp1 genomic RNA identified two mutations at nucleotides 2065 (C→T) and 2146 (C→A), predicting changes of Arg-689 to Cys and His-716 to Asn, respectively (data not shown). The mutation at nucleotide 2065 of the S glycoprotein gene is at the S1/S2 junction of the S glycoprotein; a second variant, MHV-sp2, also has been partially characterized and contained the same mutation at nucleotide 2065 (20a). The change from a positively charged amino acid to an uncharged amino acid would disrupt the highly basic N-Arg-Arg-Ala-His-Arg-C residues that act as a signal for cleavage of the 180-kDa S glycoprotein into its 90-kDa subunits (15). Six fusion-deficient MHV variants isolated from persistently infected, pri-

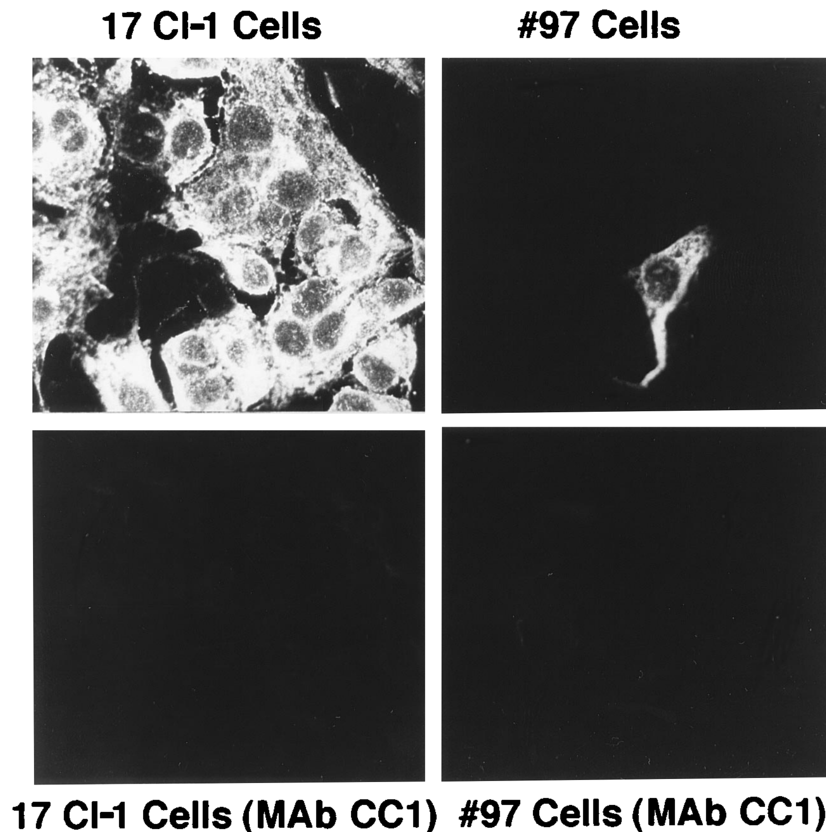

FIG. 6. Infection of #97 cells with MHV-A59 was specifically blocked by pretreatment with anti-MHVR MAb CC1. The cells were pretreated for 1 h with MAb CC1 and then inoculated with MHV-A59 in the presence of the antibody. The expression of the viral S glycoprotein at 9 h p.i. was assessed by immunofluorescence with anti-S antibody.

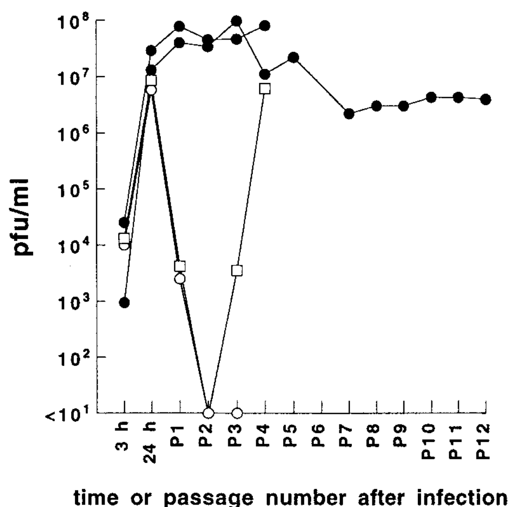

FIG. 7. #402 cells were susceptible to reinfection with MHV-A59 virus. #402 cells in 25-cm^2 flask (approximately 5×10^6) were infected with MHV-A59 (open circles and squares) or MHV-pi263 (closed circles) at 50 to 100 PFU/cell. The adsorption period was 1 h at room temperature. After the adsorption period, the virus inoculum was removed, the cells were rinsed three times with Hanks' balanced salt solution, the culture was fed with 10 ml (per flask) of growth medium containing 10 mM HEPES buffer, and the cells were incubated for 1 h (until 2 h p.i.) at 37°C and 7.5% CO_2, after which the cells were rinsed again and refed. At 3 h p.i., 1.5 ml of medium was removed and replaced. At 24 h p.i., cells were subcultured after dilution of 1:30 (P1). Every 3 days thereafter, the cells were subcultured. After the second passage (P2), one of the cultures infected with MHV-A59 (open squares) was infected again, and a portion of the medium was sampled at 3 h p.i. (P3 on the *x* axis) and at 24 h p.i. (P4 on the *x* axis). The titer was determined by plaque assay on 17C1-1 cells.

mary mouse glial cell cultures contained an alteration at the His-716 codon that predicted substitution by a negatively charged Asp residue, and such viruses were less pathogenic than MHV-A59 (8, 10). Together, such findings argue that for cleavage to occur this residue must be a positively charged amino acid or a His residue and long-term persistent infections somehow select for viruses carrying mutations in this codon.

In summary, the presence of a small fraction of 17C1-1 cells that express little or no MHVR was necessary to establish persistent MHV-A59 infection. The persistently infected cultures in turn were maintained by the continuous generation of a minor population of MHVR-expressing cells from the major population of MHVR-negative cells. These two cell populations established a dynamic equilibrium in the culture. Thus, as concluded by Mizzen et al. (17), persistence was a property of the culture and not of an individual cell. However, the regulation of the expression of the MHVR was the property of individual cells. The cellular signals regulating MHVR expression are clearly of interest as they relate to virus infectivity, persistence, and pathogenesis. That a virus uses a receptor that is not expressed on every cell in a given population is an interesting strategy that would allow the virus to infect only a subpopulation of cells and set up a persistent infection. The surviving receptor-negative cells would then generate MHVR-expressing cells at a low frequency. The ratio of receptor-positive to receptor-negative cells in a tissue or population of cells would thus be one factor that determines the extent of the tissue pathology and whether persistent infection can develop.

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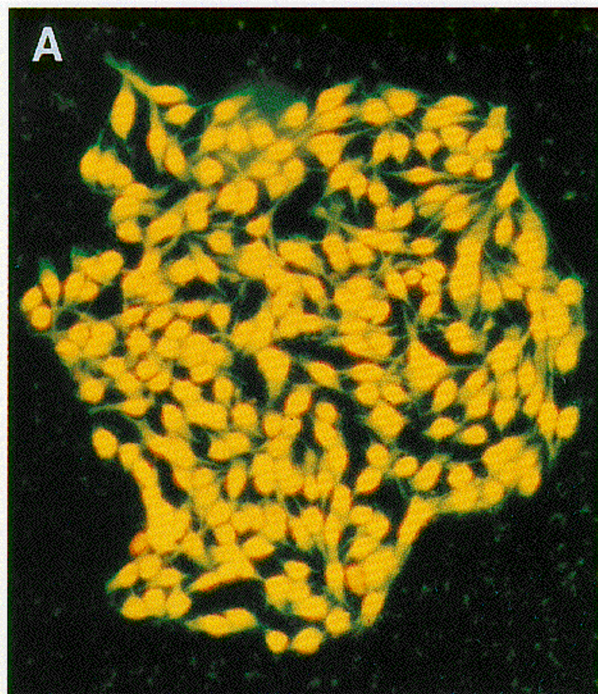

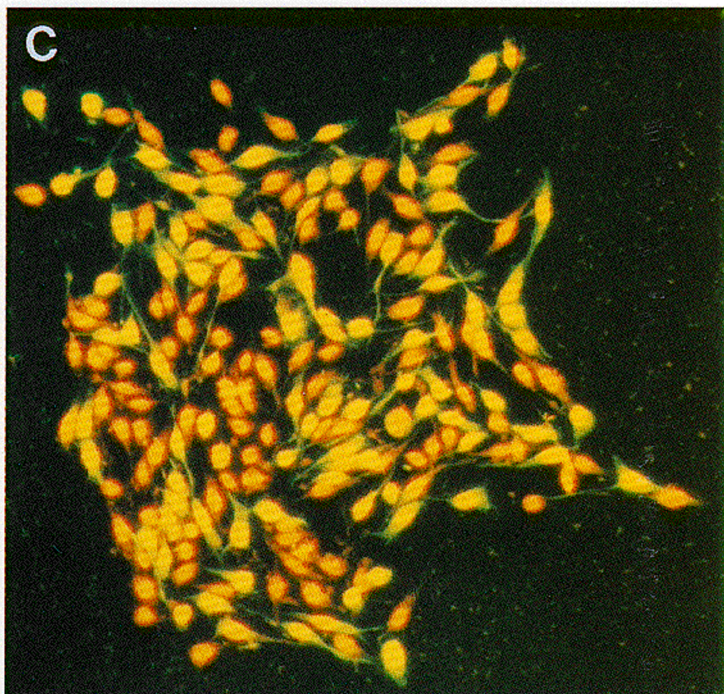

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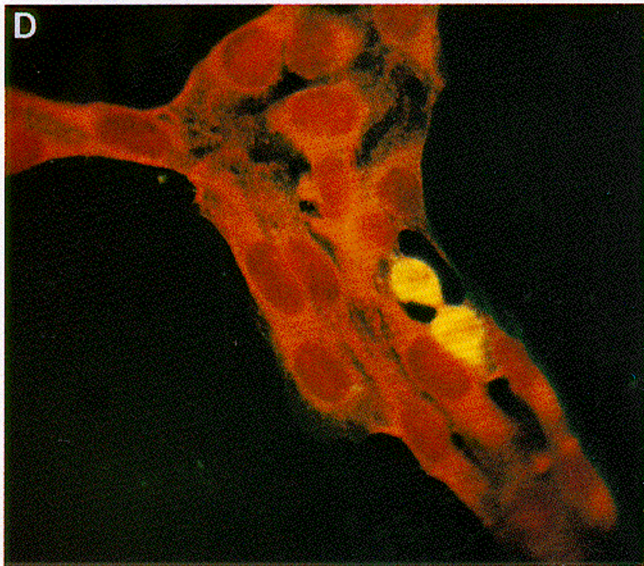
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