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Molecular Anatomy of Mouse Hepatitis Virus Persistence: Coevolution of Increased Host Cell Resistance and Virus Virulence

WAN CHEN[1] AND RALPH S. BARIC[1,2]*

Department of Epidemiology, Program in Infectious Diseases,[1] and Department of Microbiology and Immunology,[2] University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599-7400

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Persistent infection of murine astrocytoma (DBT) cells with mouse hepatitis virus (MHV) has been established. From this in vitro virus-host system, persistence is mediated at the level of cellular MHV receptor (MHVR) expression and increased virus virulence. MHV persistence selects for resistant host cell populations which abate virus replication. Reductions in MHVR expression were significantly associated with increased host resistance, and transfection of MHVR into resistant host cells completely restored the capacity of cells to support efficient replication of MHV strain A59. The emergence of resistant host cells coselected for variant viruses that had increased avidity for MHVR and also recognized different receptors for entry into resistant cells. These data illustrate that MHV persistence in vitro provides a model to identify critical sites of virus-host interaction at the cellular level which are altered during the evolution of host cell resistance to viral infection and the coevolution of virus virulence.

The mechanisms by which highly cytolytic RNA viruses establish and maintain a persistent infection in cell culture are not completely understood but likely involve the evolution of the host cell and the virus to ensure dual survival (2, 15). While down regulation of viral gene function and expression has been proposed as the predominant mechanism by which RNA viruses limit cell killing (6, 32), coevolution of the host cell and the virus has been demonstrated to function in the establishment and maintenance of foot-and-mouth disease virus (FMDV), poliovirus, reovirus, and parvovirus persistence. In these models persistence selects for resistant host cells which block virus infection or cytopathic effects, subsequently selecting for coevolving variant viruses which replicate efficiently in these cells (6, 41, 50). Unfortunately, the mechanisms of host cell resistance, subsequent virus adaptation, and coevolution in culture and their roles in persistence are poorly understood.

Mouse hepatitis virus strain A59 (MHV-A59), a member of the family *Coronaviridae*, contains a 32-kb single-stranded positive-polarity RNA genome (47). MHV entry into susceptible cells is mediated by specific interactions between the viral S glycoprotein and the cellular MHV receptor (MHVR) (18, 19). MHVR is a 110-kDa member of the biliary glycoprotein (BGP) subgroup of the carcinoembryonic antigen (CEA)-related glycoprotein (CGM) family in the immunoglobulin superfamily (18, 42). Two *Bgp* genes (*Bgp1* and *Bgp2*) have been characterized (42, 45). Two polymorphic alleles, $Bgp1^a$ and $Bgp1^b$ of the *Bgp1* gene encode numerous glycoprotein isoforms that are expressed from alternative splicing of a single gene and can serve as receptors for MHV (45). Bgp A, formerly designated mmCGM1 or MHVR1, is one of the $Bgp1^a$ gene products and is expressed in BALB/c, C57BL/6, and C3H mice (17). Bgp B, formerly designated mmCGM2 or MHVR2, is one of the $Bgp1^b$ products and is expressed in resistant SJL/J and outbred CD1 mice (17, 42, 70). BGPs may be expressed as two or four immunoglobulin C (IgC)-like intracellular domains with long or short intracytoplasmic tails. The N-terminal domains of BGPs interact with the MHV S glycoprotein to initiate infection (16, 17). Both two- and four-domain isoforms of Bgp A can serve as functional receptors for MHV-A59 (17, 70). Although Bgp B is also a functional MHV receptor, it binds virus less efficiently than the Bgp A isoforms as determined by virus overlay protein blot assays in vitro (17, 67). A novel *Bgp* gene, *Bgp2*, whose product can also serve as a receptor for MHV, although it is not as efficient as Bgp A, has been recently identified (45).

The precise mechanisms regulating MHV persistence are unclear (4, 23, 37, 60). Persistently infected cultures produce defective interfering RNAs and temperature-sensitive, fusion-defective, and small-plaque variant viruses, but these mutants are probably not critical for establishing MHV persistence (28, 30, 34, 61). Intraleader mutations and open reading frames as well as leader RNA polymorphisms which may attenuate virus gene expression, transcription, and replication evolve during the maintenance of bovine coronavirus persistence in vitro (32). We have shown that MHV persistence in vitro selects for more-virulent variant viruses containing a mutation in the genomic 5′ untranslated region (5′-UTR) which enhances expression of the MHV polymerase gene products (10). Other studies suggest that alterations in host gene expression are critical and that the epigenetic expression of the Bgp A receptor may contribute to host cell resistance and MHV persistence (54). In this study, we demonstrated that MHV persistence in vitro is mediated by the epigenetic selection for resistant host cells which express little if any Bgp A protein. In addition, MHV persistence also requires the subsequent coevolution of variant viruses which display altered avidity for Bgp A and recognize an entirely new receptor protein(s) for entry into these resistant cells. Thus, this in vitro model has shown that MHV persistence is mediated by resistance to infection at the cellular level and results in coevolution of variant viruses with increased virulence and replication efficiency.

MATERIALS AND METHODS

Viruses and cells. DBT cells were originally established from a delayed brain tumor (DBT) in a CDF1 mouse inoculated intracerebrally with the Schmidt-Ruppin strain of Rous sarcoma virus (29, 38). DBT cells were obtained from Shinji Makino (University of Texas at Austin) and were maintained within 10 passages of this progenitor stock. DBT cells were maintained in Eagle minimum essential medium containing 5% fetal calf serum and 3% newborn calf serum

* Corresponding author. Phone: (919) 966-3895. Fax: (919) 966-2089.

and supplemented with 5% tryptose phosphate broth and 1% gentamicin and kanamycin. Baby hamster kidney (BHK) cells were kindly provided by Robert E. Johnson (University of North Carolina at Chapel Hill) and maintained in alpha minimum essential medium supplemented with 7% fetal calf serum, 10% tryptose phosphate broth, and 1% penicillin and streptomycin. BHK cell lines expressing the Bgp A receptor (BHK-MHVR cells) or the Bgp B receptor (BHK-mmCGM2 cells) were kindly provided by Kathryn V. Holmes (University of Colorado Health Sciences Center). These cell lines were grown in alpha minimum essential medium containing 10% fetal calf serum, 10% tryptose phosphate broth, 1% penicillin and streptomycin, and 800 μg of Geneticin (G418-sulfate) (Sigma) per ml. MHV-A59 was propagated and plaqued in DBT cells as previously described (56). Variant viruses from DBT cultures persistently infected with MHV-A59 were collected at 119 days postinfection and plaque purified twice in DBT cells as previously described (10), and they either contain the A-to-G mutation at the nucleotide (nt) 77 locus in the 5′-UTR ($V13^+$ to $V16^+$) or are identical to MHV-A59 ($V1^-$ and $V4^-$) (10).

Uninfected DBT cells and persistently infected DBT cells at 30 days postinfection were grown in six-well plates at ca. five cells per well. Individual clones were independently isolated and subcloned twice by this technique. Sixteen cell clones (designated DBT 1 to DBT 16) were isolated from uninfected DBT cells and 18 clones (designated PDBT 1 to PDBT 18) were isolated from persistently infected cultures at 30 days postinfection.

Virus growth curves, detection of intracellular viral RNA, and virus virulence assays. Individual cell clones were seeded at densities of 2×10^5 cells per well in 24-well plates and infected with MHV-A59 or different variant viruses at a multiplicity of infection (MOI) of 5 for 1 h at room temperature. The inoculum was removed, the monolayers were washed twice with phosphate-buffered saline (PBS), and fresh medium was added. Samples of virus were harvested at different times postinfection and stored at −70°C for plaque assay. Intracellular RNA was extracted at different times postinfection by using RNA STAT-60 reagents (total RNA/mRNA isolation reagent; Tel-TEST "B," Inc., Friedwood, Tex.). Equivalent amounts of intracellular RNA were bound to nitrocellulose filters, prewashed and prehybridized as previously described, and hybridized with a radiolabeled 1.6-kb MHV-A59 *N* gene probe from the IBI 76N cDNA clone (56). The ^{32}P-radiolabeled DNA probe was synthesized by using a random primer DNA labeling system (GIBCO, BRL).

The susceptibilities of different cell clones to killing by MHV-A59 and different variant viruses were determined by using a modified virulence assay developed for FMDV (41). About 2×10^6 cells were seeded onto 60-mm-diameter dishes in triplicate and infected with wild-type MHV-A59 or variant viruses at an MOI of 5. Under these conditions, extensive cytopathology was evident in the parental DBT cells after 16 to 24 h of wild-type MHV-A59 infection. At 24 h postinfection, medium was removed from the infected cultures, and the remaining cells were trypsinized and washed twice with PBS. After centrifugation, the cells were resuspended in PBS containing trypan blue stain. The number of viable cells, as determined by dye exclusion, was counted directly under a grid and compared with the number of cells in uninfected controls.

Detection of viral antigens by immunofluorescence. Different cell clones grown on LabTek chamber slides (Nunc, Inc., Naperville, Ill.) were infected with MHV-A59 or the $V16^+$ or $V1^-$ variant virus at an MOI of 5 for 1 h at room temperature. The inoculum was removed, the cells were washed twice with PBS, and complete medium was added to the cultures. At different times postinfection, infected cells were fixed with acetone-methanol (1:1) and stored at 4°C. Fixed cells were incubated with a 1:200 dilution of a polyclonal antibody against MHV3 (kindly provided by Jean-Pierre Martin, Louis Pasteur University, Strasbourg, France) for 30 min at room temperature. After three washes with PBS, the cells were incubated with 1:100 dilution of goat anti-mouse IgG–fluorescein isothiocyanate conjugate (Sigma) for 30 min at room temperature, washed with PBS, and examined under a Nikon FXA fluorescence microscope.

Detection of Bgp A by FACS analysis. The expression of Bgp A was assessed by fluorescence-activated cell sorter (FACS) analysis using the conventional second-antibody indirect-staining method according to the procedures provided by the manufacturer (Becton Dickinson Immunocytometry Systems, San Jose, Calif.). Briefly, 10^6 cells were harvested by trypsinization, resuspended in PBS, and incubated with a 1:5 dilution of monoclonal antibody (MAb) CC1 (kindly provided by Kathryn V. Holmes, University of Colorado Health Sciences Center), which recognizes the N-terminal domain of Bgp A. After 45 min on ice, the cells were washed twice with cold PBS and incubated with either a 1:30 dilution of a sheep anti-mouse IgG R–phycoerythrin conjugate (Sigma) or a 1:25 dilution of a goat anti-mouse IgG–fluorescein isothiocyanate isomer conjugate (Sigma) for 45 min on ice. Following two washes with cold PBS, the cells were resuspended at a concentration of 2×10^6 cells per ml in 0.5 ml and analyzed with a FACScan equipped with CICERO Interface and IBM-based Cyclops Software (Becton Dickinson Immunocytometry Systems).

Stable expression of Bgp A in cell lines. The plasmid MHVR, containing the four-domain isoform of *Bgp1*^{a} under the control of the cytomegalovirus promoter in the $pcDNA_3$ vector (Invitrogen), was kindly provided by Kathryn V. Holmes (University of Colorado Health Sciences Center) and used for stable expression of Bgp A. MHVR was transfected into an MHV-A59-resistant cell clone (PDBT 5) isolated from persistently infected DBT cells at 30 days postinfection. Transfection was performed with 20 μg of the plasmid and 5×10^6 cells by the $CaPO_4$ DNA transfection method with the transfection MBS mammalian transfection kit (Stratagene) according to the manufacturer's recommendations. Individual neomycin-resistant clones were isolated after 4 weeks of G418 selection, and clones expressing Bgp A were identified by FACS analysis.

Preparation of cellular RNA and reverse transcriptase PCR (RT-PCR) amplification. Total cellular RNA was prepared from different cloned cell lines by using RNA STAT-60 reagents (total RNA/mRNA isolation reagent) according to the manufacturer's direction (Tel-TEST "B", Inc.). To detect *Bgp1* mRNA expression, 5 μg of total RNA was reverse transcribed with avian myeloblastosis virus reverse transcriptase (Promega) as previously described (55) with primer R1440(+) (TAG GAG AGT TGT CAG AAG), which is complementary to nt 1440 to 1458 at the 3′ end of the *Bgp1* gene in the intracytoplasmic tail domain (18, 42). The cDNA was amplified by PCR with the 5′ end primer R436(+) (GAT ATG ACA GAT GAA AAC TAT) (nt 436 to 453), which is in the A2a/A2b domain of *Bgp1*, or R1073(+) (CAG TCA AAG AAC TAG ACT) (nt 1073 to 1090), located in the N1/N2 domain of *Bgp1* (18, 42), and the 3′ end primer R1440(−) (see Fig. 5A). PCR was performed with *Taq* DNA polymerase (GIBCO, BRL) for 30 cycles at 94°C for 1.5 min, 45°C for 1.5 min, and 72°C for 2 min. Contamination of the RNA preparations by residual genomic DNA was detected by direct amplification with *Taq* polymerase under the same conditions.

Southern blot analysis of RT-PCR products. The PCR products were separated by electrophoresis in 1.2% agarose gels and then transferred to a Hybond-N^+ membrane (Amersham) according to the manufacturer's directions. The membrane was prehybridized at 42°C overnight in a buffer containing 0.9 M NaCl, 0.09 M sodium citrate, 1× Denhardt's solution (containing 0.2 mg of Ficoll per ml, 0.2 mg of polyvinylpyrrolidine per ml, and 0.2 mg of bovine serum albumin per ml), 0.5% sodium dodecyl sulfate (SDS), 100 μg of salmon sperm DNA per ml, and 0.05% sodium PP_i. To detect *Bgp1* expression, a ^{32}P-labeled specific *Bgp1* internal oligonucleotide probe, R1182(+) (CTC TCC CAG AAC AAC AGC ATC) (nt 1182 to 1203), located in the A2a/A2b domain and conserved in all *Bgp1*^{a} and *Bgp1*^{b} isoforms (18, 42), was hybridized with the filter at 42°C for 18 h. After hybridization, the membrane was washed with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 0.1% SDS at room temperature twice for 10 min each, then with 1× SSC containing 0.1% SDS at 42°C twice for 15 min each, and then with 0.1× SSC containing 0.1% SDS at 42°C twice for 15 min each.

Blockade of Bgp A receptor. Different cell clones were seeded at densities of 2×10^5 cells per well in 24-well plates and incubated with 200 μl of a 1:2 dilution of anti-Bgp A MAb CC1 for 1 h at 37°C. The MAb was collected hybridoma culture supernatants and was kindly provided by Kathryn V. Holmes (University of Colorado Health Sciences Center). After the incubation, the antibodies were removed and the cells were challenged with MHV-A59 or variant viruses at an MOI of 5 for 1 h at room temperature. The virus inoculate was then removed, the cultures were washed twice with PBS to remove residual virus, and 300 μl of a 1:2 mixture of MAb CC1 and complete medium was added to the cultures. Samples were harvested at different times postinfection and stored at −70°C for plaque assay in DBT cells. As a control, the cells were pretreated with an equivalent amount of a MAb with the same IgG isotype as MAb CC1 but directed against an irrelevant antigen (anti-E2 glycoprotein of Sindbis virus, kindly provided by Robert E. Johnson, University of North Carolina at Chapel Hill).

Statistical analysis. Statistical significance was determined by Student's *t* test or by the one- or two-tailed Fisher exact test (51).

RESULTS

MHV persistence selects for DBT cells which resist wild-type virus infection. Acute MHV-A59 infection in DBT cells was characterized by extensive fusion and death of approximately 95% of the cells within 24 h. Surviving cells replicated to confluence over the next 2 to 3 days, and progressively decreasing levels of virus-induced cytopathic effects and fusion were evident as the cells were passaged over the next 119 days. Virus titers approached 10^6 PFU/ml, and about 20 to 30% of the cells displayed viral antigen at 30 days postinfection (data not shown) (34). Over time, the percentage of antigen-positive cells also declined, until about 10% were positive by 119 days postinfection. To study the evolution of the surviving cell population, we cloned 16 DBT cell lines (DBT 1 to 16) from uninfected laboratory stock cell cultures and 18 persistent cell clones (PDBT 1 to 18) from persistently infected DBT cultures at 30 days postinfection. Epigenetic variability was clearly evident in our laboratory stock of DBT cells, since MHV-A59 infection in 81% of the DBT clones resulted in extensive cytopathic effects, syncytium formation, and cell death. In contrast, about 19% of the DBT cell clones (DBT 3, 7, and 8) produced little syncytium formation following MHV-A59 infection (data not shown). To determine whether the DBT cell

clones were permissive for MHV-A59 infection, virus growth curves were determined with both fusion-positive (DBT 9, 14, and 16) and fusion-negative (DBT 3, 7, and 8) cell clones (Fig. 1A). The average yields of MHV-A59 in these six DBT clones were 6.7 ± 4.4 PFU per cell at 24 h postinfection. MHV-A59 infection in fusion-positive clones resulted in virus yields of 6 to 14 PFU per cell. Although wild-type virus yields were reduced, productive infection was also detected in the fusion-negative clones, in which the virus yields ranged from 1.5 to 7.3 PFU per cell.

Surprisingly, all 18 persistent cell clones isolated at 30 days postinfection had cured virus infection, as no infectious virus, viral RNA, or viral protein was detected. MHV-A59 infection in all 18 persistent cell clones was associated with little if any cytopathology or syncytium formation (data not shown). MHV-A59 growth curves were evaluated in more detail with six persistent cell clones (PDBT 2, 3, 5, 6, 7, and 11). Statistically significant reductions in MHV-A59 replication were clearly evident in the PDBT cell clones compared with the DBT clones (Fig. 1B). Virus yields ranged from 0.02 to 1.3 PFU per cell in these six clones, with mean virus yields per cell of 0.39 ± 0.47 PFU ($P < 0.01$). MHV-A59 replication was almost completely inhibited in PDBT 5 and PDBT 11, since virus yields averaged 0.02 to 0.08 PFU per cell (Fig. 1B). These data clearly indicated that MHV persistence was associated with the evolution of host cells which resisted MHV-A59 infection and cytopathic effects in vitro.

More-virulent MHV-A59 variant viruses coevolve during persistent infection. Previous studies suggested that the selection for host cells that resisted wild-type virus infection was associated with the coevolution of more-virulent virus variants during persistence in vitro (41). We have shown that MHV persistence in vitro is associated with the evolution of a specific mutation in the 5′-UTR of the genomic RNA which enhances the replication efficiency of MHV variants isolated at 119 days postinfection (10). Therefore, we examined the replication efficiencies and virulence of variant viruses, isolated at 119 days postinfection, which contained the 5′-UTR mutation ($V13^+$ to $V16^+$) or lacked the mutation at this locus ($V1^-$ and $V4^-$) in DBT and PDBT cell clones.

The replication of variant virus $V16^+$ was extremely efficient and elicited extensive syncytium formation in all DBT cell clones, including those DBT clones that resisted wild-type MHV-A59-induced fusion and cytopathology (DBT 3, 7, and 8) (Fig. 1A). The mean yields of $V16^+$ in the DBT cell clones approached 52.4 ± 27.4 PFU per cell, which was significantly higher than those detected in the same DBT cell clones infected with the parental MHV-A59 virus (6.7 ± 4.4) ($P < 0.001$). Similar results were also obtained with the $V1^-$ virus (data not shown). Viral cytopathology and cell killing assays also demonstrated that the $V1^-$ and $V16^+$ variant viruses were more cytolytic than MHV-A59. For example, fewer than 1% of DBT 9 cells survived $V1^-$ or $V16^+$ infection, compared with 4.2% cell survivors following parental MHV-A59 infection ($P < 0.01$) (Table 1). Other variant viruses ($V4^-$, $V13^+$, $V14^+$, and $V15^+$) isolated at 119 days postinfection also displayed increases in virulence and viral replication similar to those shown by $V1^-$ and $V16^+$ in DBT 9 cells (Table 1). These data strongly imply that variant viruses which were more virulent and replicated more efficiently in DBT cells cloned from our original laboratory stock have emerged by 119 days postinfection.

We next examined the replication efficiencies and virulence of these variant viruses in PDBT cell clones isolated at 30 days postinfection. Although all persistent cell clones were permissive for $V16^+$ virus replication, viral yields were reduced three- to fourfold compared with the maximal yields detected in DBT cell clones ($P < 0.05$) (Fig. 1B and Table 1). In PDBT clones, $V16^+$ virus replication was also more efficient than parental virus replication, since the yields of $V16^+$ and the parental MHV-A59 averaged 13.9 ± 10.2 and 0.39 ± 0.47 PFU per cell, respectively ($P < 0.01$). To determine whether this phenotype was characteristic of other variant viruses isolated at 119 days postinfection, the replication efficiencies of five additional variant viruses, containing the 5′-UTR mutation ($V13^+$, $V14^+$, and $V15^+$) or containing the wild-type sequence at this locus ($V1^-$ and $V4^-$), were examined in DBT 9 and PDBT 5 cells. All variant viruses replicated to significantly higher titers than the parental MHV-A59 in both cell clones (Table 1) ($P < 0.001$). In addition, the replication of these viruses in DBT cells was also more efficient than that in PDBT clones ($P < 0.05$). To determine whether the variant viruses were more virulent than MHV-A59 in persistent cell clones, PDBT 5 cells were infected with MHV-A59, $V1^-$, or $V16^+$. At 24 h postinfection, more than 95% of the PDBT 5 cells had survived MHV-A59 infection. Under identical conditions, only 66 and 69% of the PDBT 5 cells had survived $V1^-$ and $V16^+$ infection, respectively (Table 1). Immunofluorescence assays at 7 h postinfection indicated that MHV-A59 infected fewer than 1% of PDBT 5 cells, compared with 15 to 20% for $V1^-$- or $V16^+$-infected cultures (Fig. 2B and C).

Since replication of wild-type MHV-A59 was dramatically reduced in the PDBT clones, we next determined whether the reduction in virus yields occurred prior to or after virus transcription. Cultures of PDBT 5 cells were infected with MHV-A59, and intracellular RNA was isolated at different times postinfection, bound to nitrocellulose filters, and probed with an MHV-A59 *N* gene cDNA probe (56). Significant amounts of viral RNA synthesis were detected in MHV-A59-infected DBT 9 cells but not in the MHV-A59-infected PDBT 5 cells, suggesting that the replication of MHV-A59 was inhibited at an early stage prior to RNA synthesis (Fig. 3). Immunofluorescence assays also confirmed this observation (Fig. 2B and C). These data were consistent with the hypothesis that more-virulent variant viruses had evolved during MHV persistence in vitro which could overcome the early block of MHV-A59 infection exhibited by persistent cell clones.

Host cell resistance is associated with reductions in *Bgp1* expression. Although selection for host cells that resist viral infection during MHV, reovirus, poliovirus, and FMDV persistence has been reported, their role in persistence has not been elucidated, and the genetic basis for host cell resistance is not clear (6, 15, 41, 54). Since our data had suggested that resistance to MHV-A59 infection in PDBT cell clones occurred at a early step in the virus replication cycle, and since previous studies also suggested that Bgp A (MHVR) expression was reduced during MHV persistence in 17CL-1 cells (54), the levels of *Bgp1* expression in the cloned cell lines were examined. The relative levels of *Bgp1* mRNA and protein expression in DBT and PDBT cell clones were quantitated by RT-PCR and by FACS analysis with MAb CC1, which recognizes the N-terminal domains of both the two- and four-domain isoforms of Bgp A. As expected, over 77% of the DBT 9 cells were Bgp A positive by FACS analysis, with a mean fluorescence of 6.62 after subtraction of background fluorescence (Fig. 4A). In contrast, the mean fluorescence of PDBT 5 was only 0.26, and it was almost equivalent to that of unstained control cells (Fig. 4E). Since these data suggested that the absence of Bgp A may contribute to the resistance to wild-type MHV-A59 infection, we compared the expression of Bgp A in six additional cell clones. DBT 8 (fusion negative) and DBT 14 (fusion positive) were permissive for MHV-A59 infection (Fig.

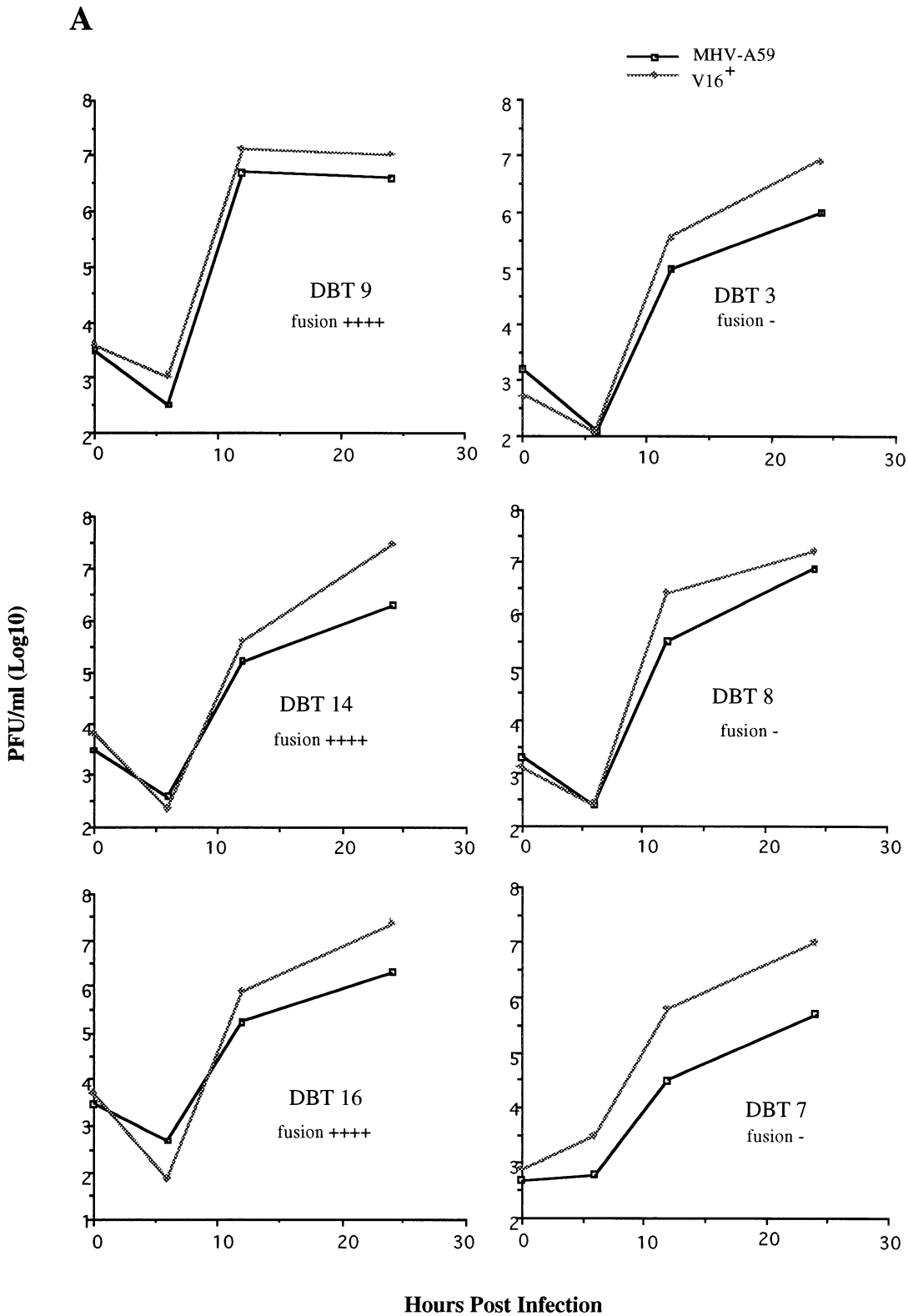

FIG. 1. Growth of MHV-A59 and V16$^+$ in DBT and PDBT cell clones. Individual clones of DBT cells (A) or PDBT cells (B) were isolated as described in Materials and Methods. Cultures (2×10^5 cells) of cloned DBT or PDBT cells were infected with parental or variant viruses at an MOI of 5. Virus samples were collected at different times postinfection, and titers were determined on DBT cells by plaque assay.

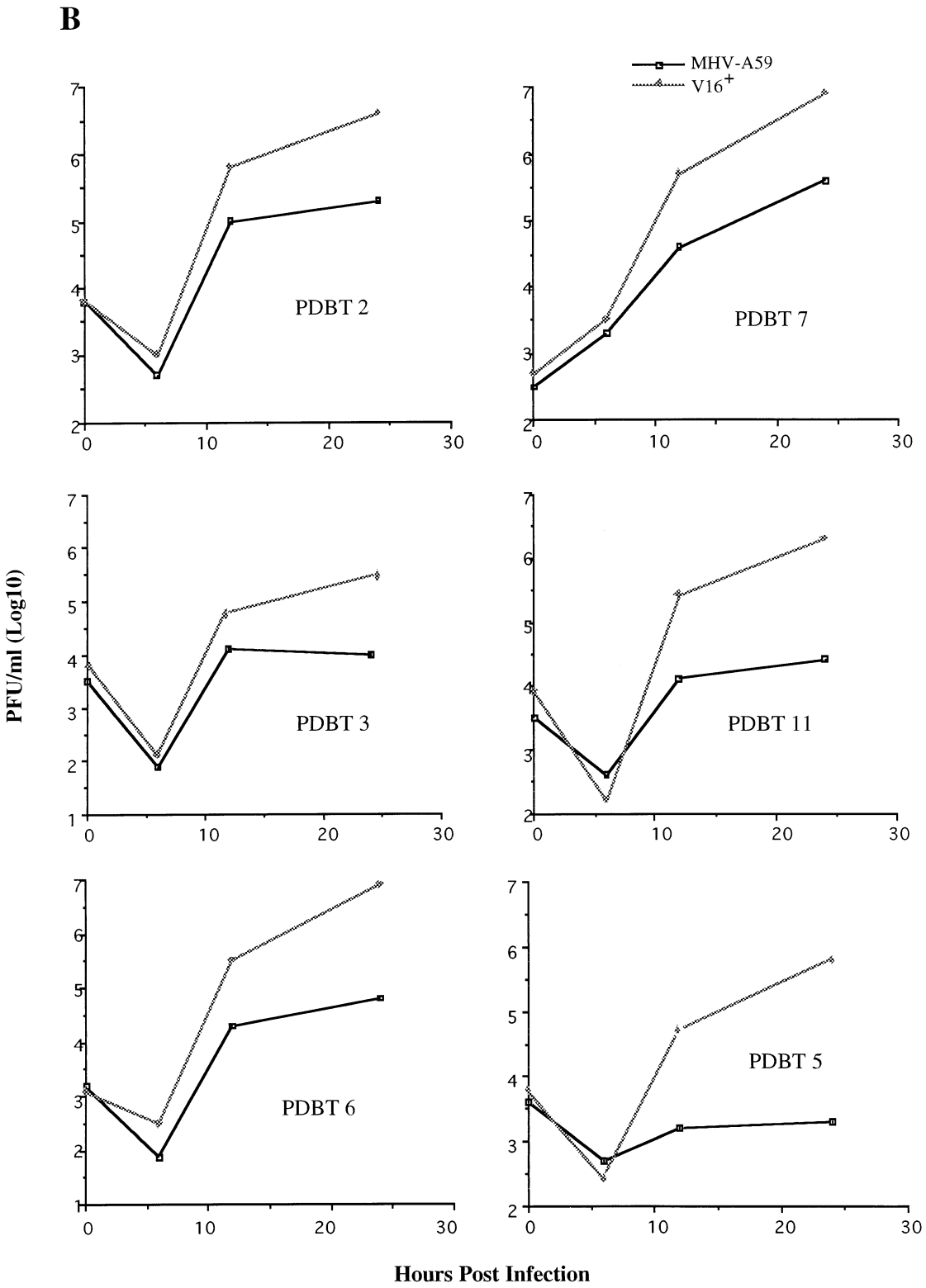

FIG. 1—*Continued.*

1). Both cell clones clearly expressed high levels of Bgp A compared with an unstained control (Fig. 4B and C), with a mean fluorescence of 4.14 and 11.60, respectively. It was interesting that the expression of Bgp A in fusion-negative clone 8 was lower than that detected in fusion-positive clones (DBT 9 and DBT 14). In contrast to the DBT cell clones which expressed high levels of the receptor, all PDBT clones examined were Bgp A negative by FACS analysis, with mean fluores-

TABLE 1. Virulence and yields of MHV-A59 and variant viruses in DBT 9 and PDBT 5 cell clones at 24 h postinfection

Virus	% Cell survival (mean ± SD)		Virus yield (PFU/cell)	
	DBT 9	PDBT 5	DBT 9	PDBT 5
A59	4.20 ± 0.35	94.7 ± 1.4	14.3	0.02
$V16^+$	0.20 ± 0.15	68.7 ± 2.5	36.7	10.00
$V4^-$	0.80 ± 0.17	ND[a]	39.0	10.00
$V1^-$	0.30 ± 0.06	66.3 ± 3.0	33.0	11.30
$V13^+$	0.25 ± 0.07	ND	58.0	14.70
$V14^+$	0.53 ± 0.12	ND	26.0	15.80
$V15^+$	0.73 ± 0.11	ND	32.0	15.00

[a] ND, not determined.

cences of 1.01, 0.32, 0.10, and 0.83 in PDBT clones 3, 2, 7, and 11, respectively (Fig. 4E to I). After adjustment for background staining levels, an approximate 10- to 20-fold decrease in Bgp A expression was evident in persistent cell clones compared with DBT cell clones.

To confirm the reduction in Bgp A expression in these cell clones, levels of *Bgp1* mRNA were detected by using RT-PCR, a more sensitive technique (53). Intracellular RNA was isolated from DBT and PDBT cell clones, and cDNA was synthesized by using a Bgp-specific oligonucleotide primer, 1440(−), as described in Materials and Methods. Two specific sets of primer pairs, (i) 436(+) and 1440(−) and (ii) 1073(+) and 1440(−), spanning nt 436 to 1140 and nt 1073 to 1440 in Bgp A, respectively, were used for PCR amplification (Fig. 5A) (18, 42). The products were separated on agarose gels, bound to nitrocellulose filters, and probed with a ^{32}P-labeled specific internal primer, 1182(+) (nt 1182 to 1203 in Bgp A) (Fig. 5A) (18, 42). With the 1073(+) and 1440(−) primers, which reside within the A2a/A2b domain and intracytoplasmic tails, respectively, all $Bgp1^a$ and $Bgp1^b$ isoforms will result in a 370-bp product following PCR amplification (Fig. 5A). In contrast, with the 436(+) and 1440(−) primer pair, four-domain isoforms of $Bgp1^a$ or $Bgp1^b$ will result in an ~1.0-kb PCR product, while two-domain isoforms will result in a 440-bp product (42). By RT-PCR, expression of BGP mRNA was clearly evident in all DBT cell clones (DBT 8, 9, and 14), consistent with our FACS results. Interestingly, the two-domain isoforms of BGP were predominantly expressed in DBT cell clones, since the 440-bp product was preferentially detected in these gels (Fig. 5B, lanes 1). In the absence of reverse transcription, no BGP products were detected following *Taq* amplification, ruling out the possibility of genomic DNA contamination in our mRNA preparations (data not shown). Importantly, no significant levels of *Bgp1* mRNA expression were detected in persistent cell clones (PDBT 2, 3, 5, 7, and 11) (Fig. 5B), consistent with previous findings that MHV-A59 persistence in vitro selected for host cell populations that expressed little if any $Bgp1^a$ or $Bgp1^b$

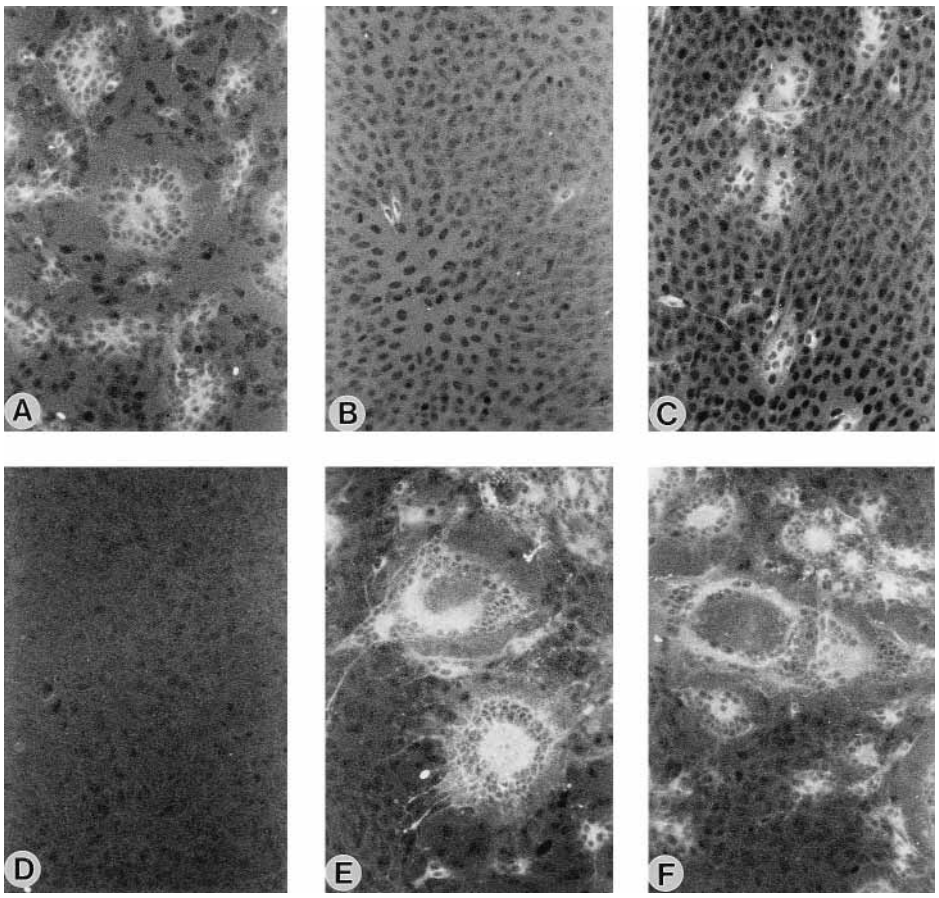

FIG. 2. MHV-A59, $V16^+$, or $V1^-$ viral protein expression in different cell clones. Cultures of cells were infected with MHV-A59, $V16^+$, or $V1^-$ and fixed as described in Materials and Methods at different times postinfection. Viral antigen expression was detected by immunofluorescence assay with anti-MHV polyclonal serum. (A and B) MHV-A59 infection in DBT 9 (A) or PDBT 5 (B) cells at 7 h postinfection. (C) $V16^+$ infection in PDBT 5 cells at 7 h postinfection. (D to F) MHV-A59 (D), $V16^+$ (E), and $V1^-$ (F) viral antigen detected at 24 h postinfection in DBT 9 cells pretreated with MAb CC1.

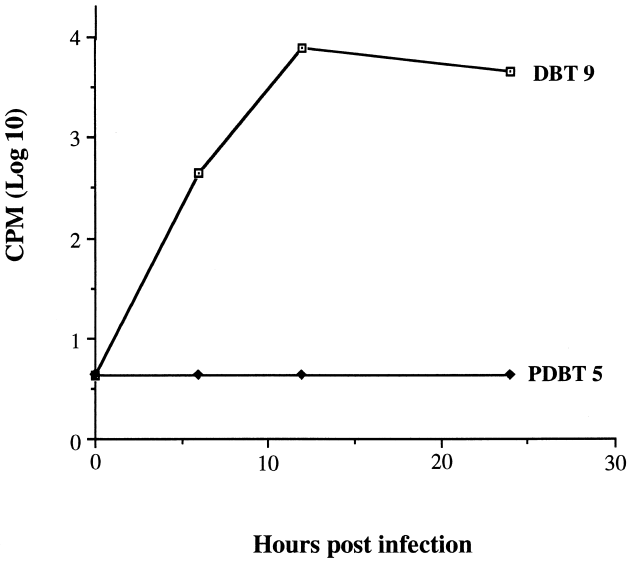

FIG. 3. Intracellular viral RNA detected in DBT 9 and PDBT 5 cell clones infected with MHV-A59. Cells were infected with MHV-A59 at an MOI of 5, and intracellular RNA was extracted at different times postinfection as described in Materials and Methods. Equivalent amounts of intracellular RNA were bound to nitrocellulose filters, and viral RNA was detected with a ^{32}P-radiolabeled MHV-A59 *N* gene probe. The dots were localized, excised, and counted after the filters were exposed to Kodak X-ray film, and the amount of RNA is reported as counts per minute.

products. Statistical analysis also confirmed that significant differences in Bgp1 expression between the populations of PDBT and DBT cell clones were evident ($P < 0.05$). These data argued that MHV infection eliminated BGP-expressing cell populations during acute infection and selected for BGP-negative cells that could survive and proliferate in the presence of virus.

Mechanisms of cell resistance to MHV-A59 infection. To confirm the hypothesis that persistent cell clones resisted MHV-A59 infection by reducing or abating BGP expression (54), a plasmid expressing the four-domain Bgp A (MHVR) under the control of a cytomegalovirus promoter in the pcDNA3 vector was transfected into PDBT 5 cells to obtain cell lines that stably express Bgp A (18). Following transfection, neomycin-resistant cell clones were isolated, and FACS analysis with MAb CC1 was used to determine relative levels of Bgp A expression. Several G418-resistant clones (designated PDBT 5 R-1 to PDBT 5 R-24) were isolated, in which Bgp A expression levels ranged from 4.97 to 429.39 (mean of fluorescence after removal of background). Since previous studies with alphavirus have demonstrated that levels of receptor expression were critical elements in regulating virus-receptor interaction and infectivity (65), all additional studies were performed with the PDBT 5 R-14 clone, which expressed levels of Bgp A similar to those of DBT cell clones. The PDBT 5 R-14 cells had a mean fluorescence of 4.97, compared with 0.26 in the parental PDBT 5 cells and 6.62 in DBT 9 (Fig. 4D). The PDBT 5 R-14 clone was clearly not an epigenetic variant existing within the PDBT 5 cell population, since RT-PCR analysis demonstrated that the four-domain isoform of Bgp A was expressed after transfection of Bgp A, unlike the case for all

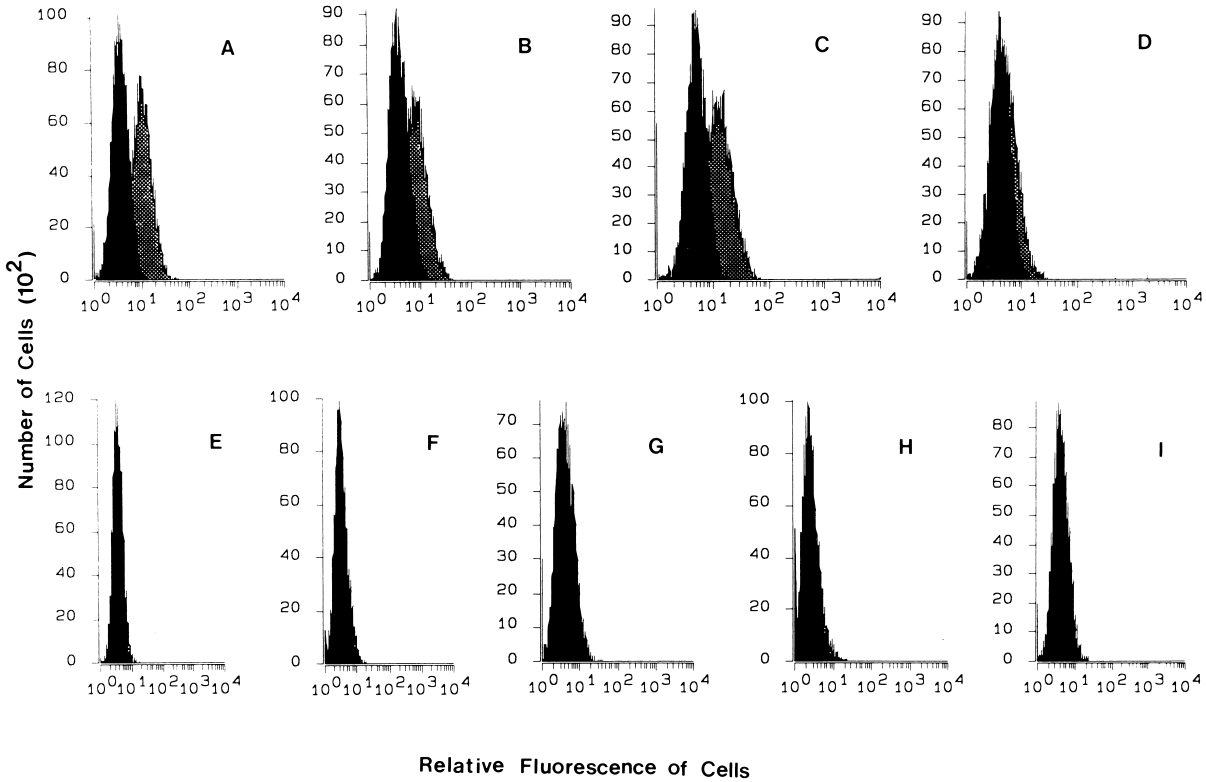

FIG. 4. Expression of Bgp A in DBT and PDBT cell clones. Levels of Bgp A expression were analyzed by FACS after staining with MAb CC1 as described in Materials and Methods. (A to C) Expression of Bgp A in DBT 9, 8, and 14, respectively; (E to I) expression of Bgp A in PDBT 5, 3, 2, 7, and 11, respectively; (D) expression of Bgp A in PDBT 5 R-14.

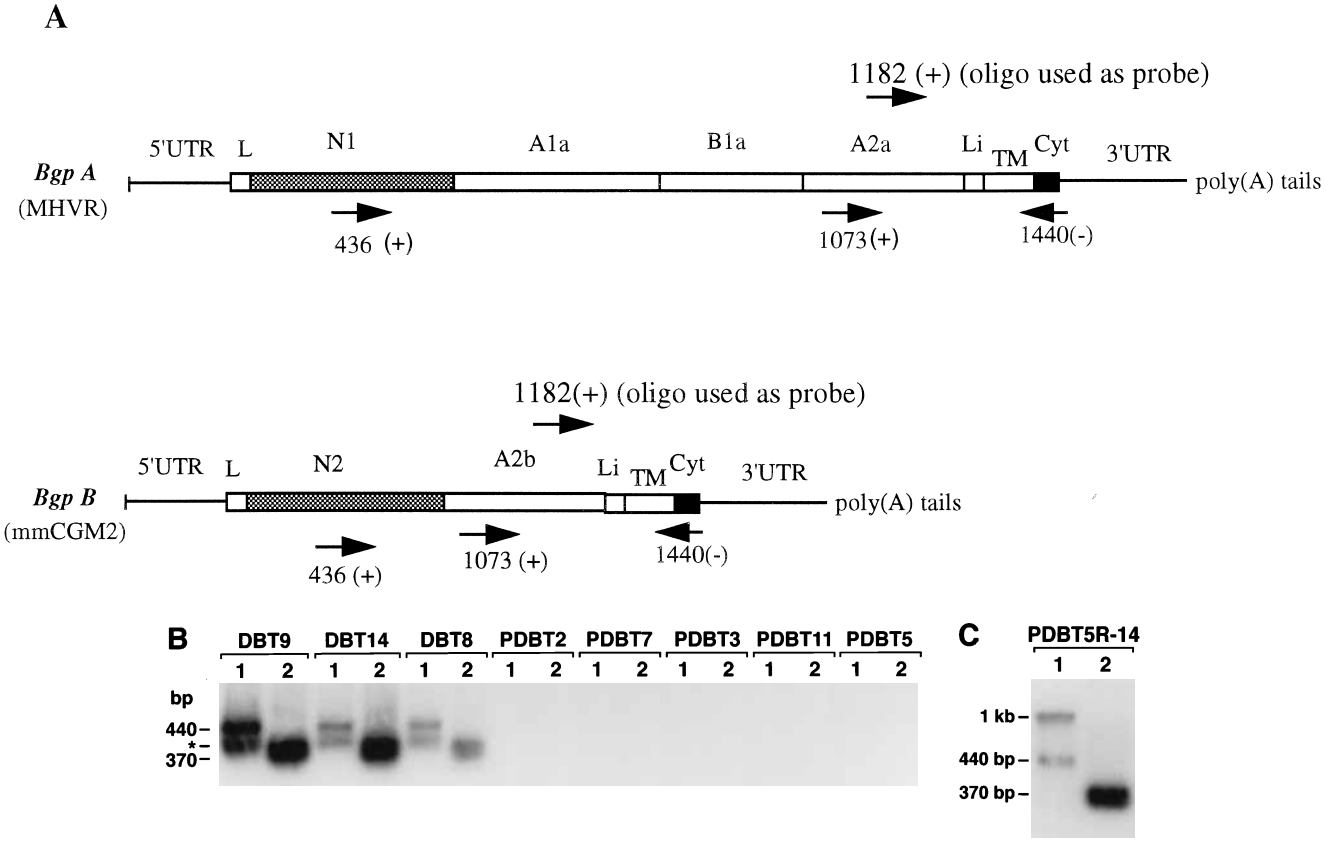

FIG. 5. Detection of *Bgp1* mRNA in DBT and PDBT cell clones. Cellular RNA was isolated from individual cell clones, and RT-PCR was performed with *Bgp1*-specific primers (A). The PCR products were detected by using a ^{32}P-labeled *Bgp1*-specific internal oligonucleotide probe, 1182(+) (A). (B and C) Expression of *Bgp1* mRNA in DBT and PDBT cell clones (B) and PCR products detected in the PDBT 5 R-14 clone under identical experiment conditions (C). Lanes 1, cDNA amplified with the 436(+) and 1440(−) primers; lanes 2, cDNA amplified with the 1073(+) and 1440(−) primers. *, other *Bgp* alleles detected in DBT cell clones but not expressed in PDBT cell clones.

tested DBT cell clones (Fig. 5C). Following infection of PDBT 5 R-14 with the parental MHV-A59, high percentages of cells (>80%) were antigen positive by immunofluorescence assay and extensive syncytium formation (>70%) was observed in the cultures. Replication of MHV-A59 was completely restored and was nearly equivalent to levels of V16$^+$ and V1$^-$ variant virus replication in these cells at 24 h postinfection (Fig. 6A). Significant levels of virus replication and syncytium formation were also evident in all other PDBT 5 R clones that expressed similar levels of Bgp A (data not shown). In contrast, less than 1% of the MHV-A59-infected PDBT 5 cells were viral antigen positive, and virus yields were 3 to 4 log units lower. Importantly, a G418-resistant clone (PDBT 5 R-22), which was Bgp A negative by FACS analysis, was as resistant as its parental PDBT 5 clone to MHV-A59 infection (Fig. 6B). In addition, MAb CC1 completely inhibited MHV-A59 but not V16$^+$ and V1$^-$ replication in PDBT 5 R-14 cells (Fig. 6C). These data clearly demonstrate that resistance in persistent cell clones is mediated by low levels of BGP expression.

Mechanisms of increased variant virus virulence during persistent infection. Findings in our laboratory and others have demonstrated that MHV-A59 persistence in vitro was associated with the emergence of host cells that resisted wild-type virus infection by down regulation of BGP expression (54). However, we have also shown that variant viruses which replicate more efficiently in both sensitive and resistant cells emerge during MHV persistence. Since Bgp A is either not expressed or expressed at extremely low levels in the resistant cells, it seems likely that the variant viruses contain mutations which either confer an altered avidity for Bgp A or permit recognition of an alternative receptor(s) for entry into these cells. To test this hypothesis, we performed blockade experiments to determine whether entry of MHV-A59 or variant viruses could be abated by MAb CC1. Cultures of DBT 9 cells were pretreated with MAb CC1 for 1 h, and then the antibodies were removed and the cells were infected with MHV-A59, V1$^-$, or V16$^+$ at an MOI of 5. The variant viruses V1$^-$ and V16$^+$ replicated efficiently in DBT 9 cells either untreated or pretreated with MAb CC1, yet the replication of MHV-A59 was completely blocked and was reduced by over 6 log units in cells pretreated with MAb CC1 (Fig. 7A). Pretreatment with antibodies reduced the replication of variant viruses by about 1 to 2 log units compared with that in the untreated controls and reduced the number of infected DBT 9 cells from 99 to 52% as detected by immunofluorescence. The detection of viral antigens by immunofluorescence assay at 24 h postinfection also confirmed a complete block in MHV-A59, but not in V1$^-$ or V16$^+$, viral protein expression (Fig. 2A, D, E, and F). These data suggest that the variant viruses must have increased avidity for Bgp A, recognize different domains in Bgp A that are not accessible to MAb CC1 blockade, and/or recognize a different receptor(s) for entry into resistant cell clones.

We next determined whether Bgp A (MHVR) functions as a receptor for entry of the variant viruses. Previous studies

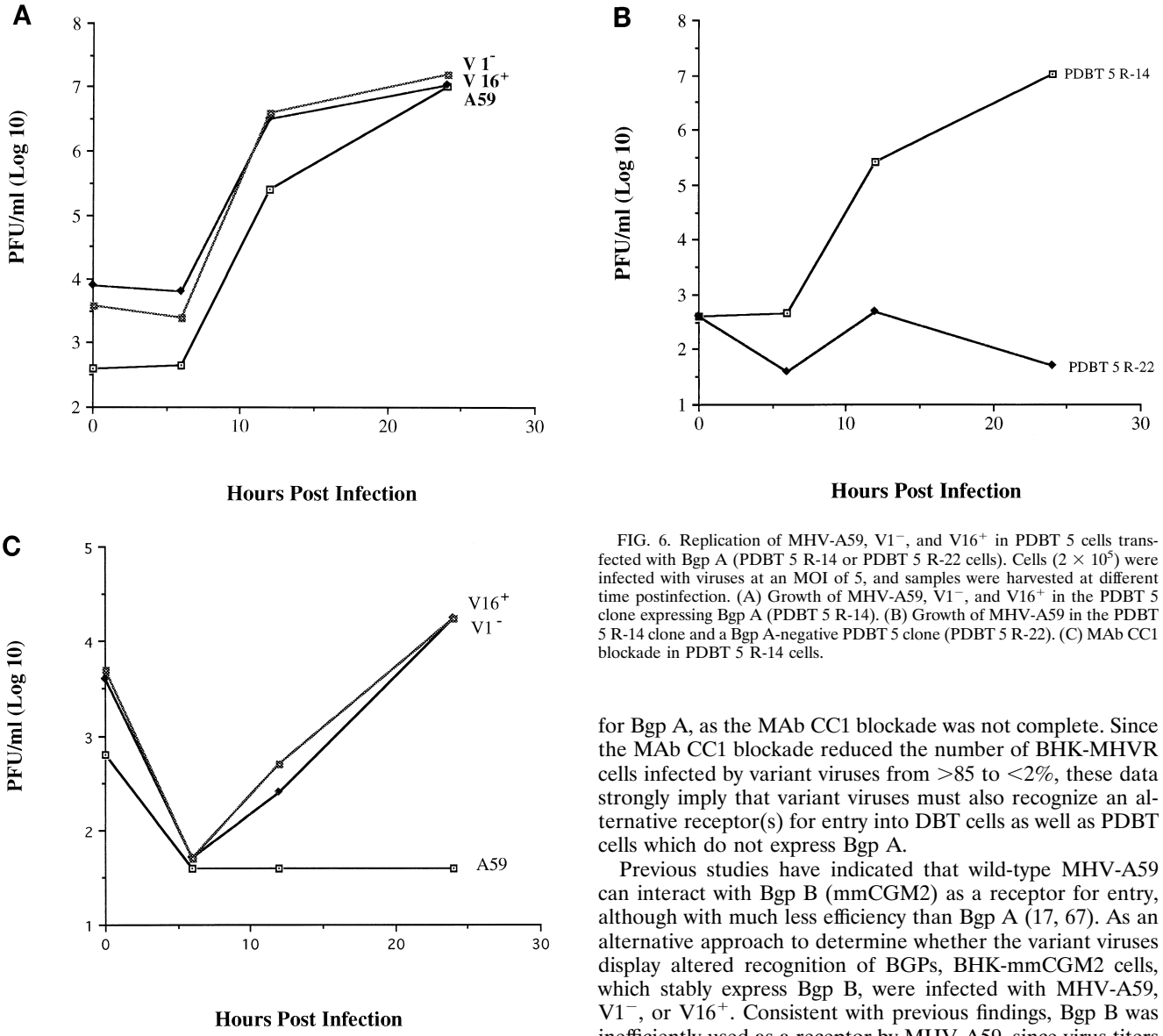

FIG. 6. Replication of MHV-A59, V1$^-$, and V16$^+$ in PDBT 5 cells transfected with Bgp A (PDBT 5 R-14 or PDBT 5 R-22 cells). Cells (2×10^5) were infected with viruses at an MOI of 5, and samples were harvested at different time postinfection. (A) Growth of MHV-A59, V1$^-$, and V16$^+$ in the PDBT 5 clone expressing Bgp A (PDBT 5 R-14). (B) Growth of MHV-A59 in the PDBT 5 R-14 clone and a Bgp A-negative PDBT 5 clone (PDBT 5 R-22). (C) MAb CC1 blockade in PDBT 5 R-14 cells.

have clearly established that MHV cannot replicate in BHK cells unless these cells express Bgp A (18). BHK-MHVR cells, which stably express Bgp A, were infected with MHV-A59, V1$^-$, or V16$^+$. Although the MHV-A59, V1$^-$, and V16$^+$ viruses could not replicate in BHK cells (data not shown), they replicated efficiently in BHK-MHVR cells, approaching titers of 10^8 PFU/ml within 24 h. These data clearly demonstrated that the variant viruses were capable of recognizing Bgp A as a functional receptor (Fig. 7B). To study whether the variant viruses were also capable of recognizing a different receptor(s) or displayed altered binding to Bgp A, blockade experiments were performed with BHK-MHVR cells. The blockade of MAb CC1 completely arrested the replication of the parental MHV-A59 in BHK-MHVR cells (>6 log units). Also, replication of the V1$^-$ and V16$^+$ variant viruses was reduced by about 4 to 5 log units compared with controls (Fig. 7B). These data suggest that the variant viruses and MHV-A59 probably recognize a similar domain in Bgp A which is accessible to MAb CC1 but that variant viruses probably display a greater avidity for Bgp A, as the MAb CC1 blockade was not complete. Since the MAb CC1 blockade reduced the number of BHK-MHVR cells infected by variant viruses from >85 to <2%, these data strongly imply that variant viruses must also recognize an alternative receptor(s) for entry into DBT cells as well as PDBT cells which do not express Bgp A.

Previous studies have indicated that wild-type MHV-A59 can interact with Bgp B (mmCGM2) as a receptor for entry, although with much less efficiency than Bgp A (17, 67). As an alternative approach to determine whether the variant viruses display altered recognition of BGPs, BHK-mmCGM2 cells, which stably express Bgp B, were infected with MHV-A59, V1$^-$, or V16$^+$. Consistent with previous findings, Bgp B was inefficiently used as a receptor by MHV-A59, since virus titers only approached 10^4 PFU/ml after 24 h of infection and virus yields were only about 0.5 PFU per cell. Under identical conditions, Bgp B was efficiently utilized as a receptor by V1$^-$ and V16$^+$ viruses and replicated to a 3- to 4-log-higher titer than MHV-A59 (Fig. 7C). Peak V1$^-$ and V16$^+$ virus yields reached 30 and 13 PFU per cell, respectively. By immunofluorescence assay, the number of BHK-mmCGM2 cells infected by the V1$^-$ and V16$^+$ viruses (66 and 49%, respectively) was significantly higher than the number of the same cells infected by MHV-A59 (<2%). Although binding assays will be needed to prove this hypothesis, these data strongly imply that the variant viruses contain mutations which permit an altered, more efficient interaction with the Bgp B receptor.

If MHV persistence in vitro selects for more-virulent viruses that are capable of infecting resistant cells which express low levels of BGP and if this is a critical element for regulating persistence, then the majority of the persisting virus should display this altered phenotype. To test this hypothesis, we examined the receptor specificities of four additional variant viruses (V4$^-$, V13$^+$, V14$^+$, and V15$^+$) isolated from persistently infected cultures at 119 days postinfection. DBT 9 cells

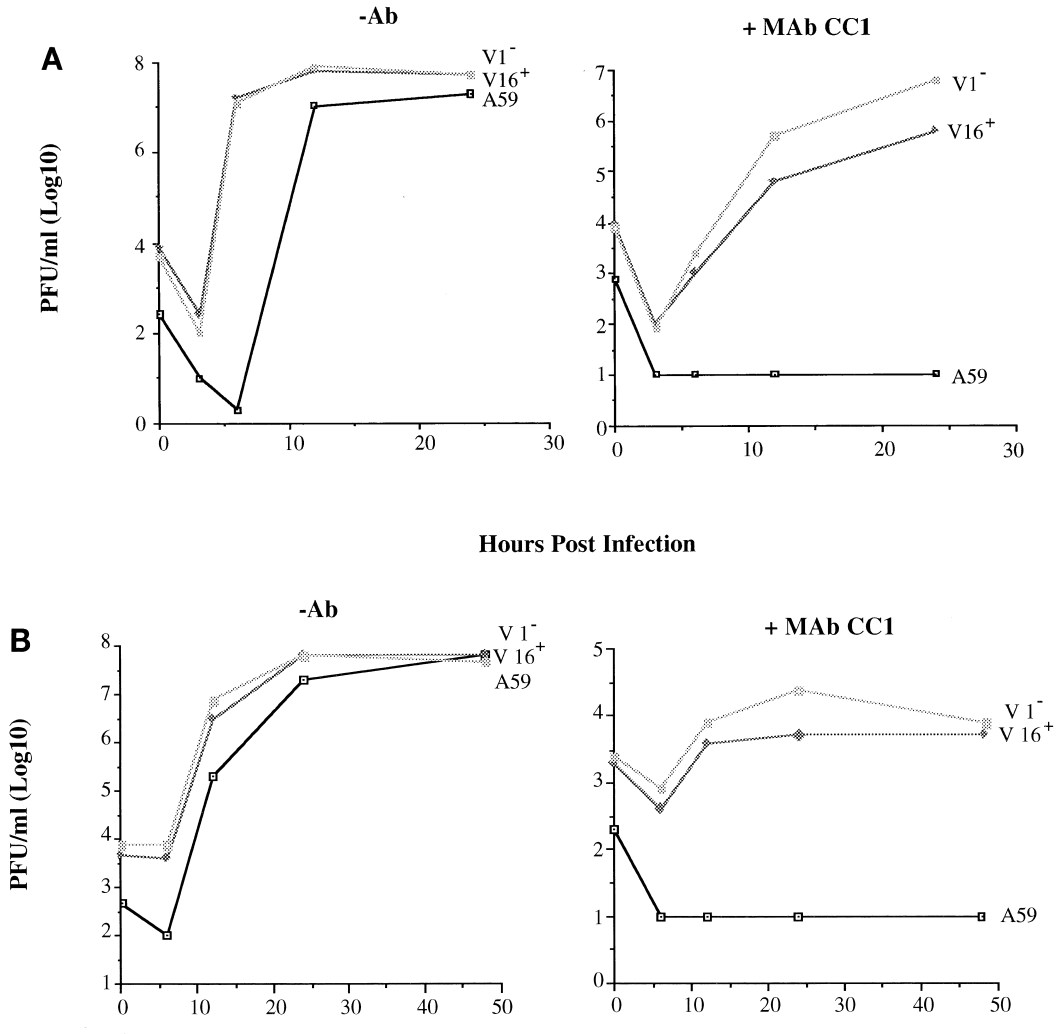

FIG. 7. Receptor specificities of variant viruses. Cultures of DBT 9 (A), BHK-MHVR (B), and BHK-mmCGM2 (C) cells were seeded at 2×10^5 cells per well and infected with MHV-A59, $V1^-$, and $V16^+$ at an MOI of 5 for 1 h. The pretreatment of cells with MAb CC1 was performed as described in Materials and Methods.

were pretreated with MAb CC1 as described above and infected with the four variant viruses. As in the case of the $V1^-$ and $V16^+$ variant viruses, MAb CC1 pretreatment failed to inhibit the replication of all other variant viruses in DBT 9 cells (Fig. 8). Taken together, our data argue that MHV persistence in vitro is mediated by the selection of resistant host cells that display reduced levels of *Bgp1* expression and by the subsequent coevolution of more-virulent variant viruses which display altered BGP receptor avidities and use an alternative receptor(s) for entry into resistant cells.

DISCUSSION

Roles of the host cell and coevolving virus in MHV persistence. The recent demonstration of persisting coronaviruses in mouse and primate central nervous systems and in the brains

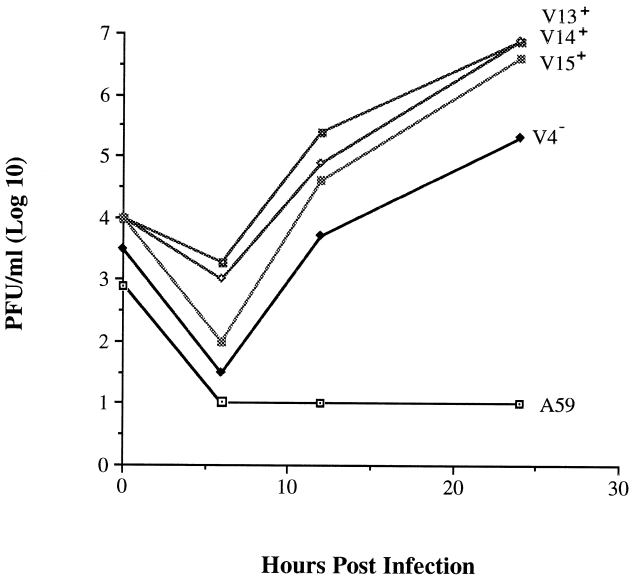

FIG. 8. Replication of MHV-A59 and variant viruses $V4^-$, $V13^+$, $V14^+$, and $V15^+$ in the presence of MAb CC1 in DBT 9 cells. Cultures of 2×10^5 cells were pretreated with MAb CC1 for 1 h and infected with viruses at an MOI of 5. Viruses were harvested at different time postinfection and titrated on DBT cells.

of multiple sclerosis patients has increased interest in identifying the critical sites of host-virus interaction which regulate the establishment and maintenance of coronavirus persistence (9, 23, 44, 49). MHV-A59 produces an acute cytolytic infection in DBT cells, yet persistent infections are readily established, providing a reproducible model of a naturally occurring virus infection (30, 34). In the absence of prior exposure to virus, cloned DBT cell lines not only displayed heterogeneous levels of *Bgp1* expression but also displayed different susceptibilities to wild-type MHV-A59 infection. This is not surprising, since a similar variability has also been reported with other cell lines in culture and since clonal expansion of individual cells in BHK-21 and 17CL-1 cell populations occurred during FMDV and MHV-A59 persistence (41, 54). The generation of cell diversity may also be favored in transformed cell lines because of their inherent genetic instability (46). After establishing MHV-A59 persistent cultures in 17CL-1 cells, Sawicki et al. (54) demonstrated the emergence of resistant host cell variants at passages 97 and 402 (about 290 and 1,200 days postinfection, respectively). In our studies, we have demonstrated that as early as 30 days postinfection, resistant cells which expressed little if any Bgp A receptor protein emerged. The rapid emergence of resistant DBT populations within 30 days postinfection, coupled with the findings of resistant 17CL-1 cells at 3 years postinfection (54), argues that host cell resistance is probably the critical element that functions in both the establishment and maintenance of MHV persistence in vitro. Cell resistance is mediated at the level of entry, since transfection and expression of Bgp A in resistant cells restored a phenotype of susceptibility to wild-type infection. However, we cannot rule out the possibility that some resistant host cells that arrest viral infection at other stages in the growth cycle may also arise during persistence, since other sites of virus-host interaction may also affect MHV entry, transcription, assembly, and release (69). Since a small percentage of the DBT cell population (~4%) survives acute MHV-A59 infection, it seems unlikely that host cell resistance resulted from mutational processes attributed to the virus. Rather, resistant cells were probably selected from preexisting host cell variants present within the cell population. In our model a small percentage (<1%) of the cloned resistant cells (PDBT 5 cells) remained susceptible to wild-type infection, consistent with the hypothesis that epigenetic variation contributed to ongoing MHV persistence in vitro (54). However, our results also reveal that the major mechanism for MHV persistence in vitro involves not only the selection for resistant host cells but also the selection for more-virulent viruses.

Rapid RNA virus evolution is a hallmark of persistent infections in vitro and in vivo (1, 7, 12, 32), yet it has been suggested that MHV persistence is uncoupled from the emergence of mutant viruses in vitro (43, 54). We disagree with this hypothesis and argue that MHV persistence in DBT cells is dependent upon the coevolution of virus. We have clearly demonstrated that MHV-A59 persistence in DBT cells selected for more-virulent variant viruses which displayed significantly increased lethality in both DBT and persistent cell clones. It is interesting to note that variant viruses isolated from persistently infected 17CL-1 cells also replicated more efficiently than wild-type virus in parental as well as in persistent cell clones (54), strongly suggesting that similar coevolutionary mechanisms may also occur in this model. Although the exact genotypic alterations regulating increased MHV virulence in cell culture are unclear, the variant viruses described in this paper are the first clearly defined MHV mutants that displayed altered receptor specificity and avidity and cell tropisms in vitro. Mutations which alter binding and/or increased avidity for Bgp A might evolve and permit infection of cells expressing marginal levels of *Bgp1*. Alternatively, variant viruses might recognize a new receptor(s) for entry into resistant cells, or both mechanisms might evolve during MHV persistence in vitro. This would not be unprecedented, since enterotropic strains of MHV probably recognize native receptors other than Bgp A for entry (11) and human immunodeficiency virus recognizes at least two different receptors for entry into cells (21, 69). Our findings are also consistent with the hypothesis that neurotropic strains of MHV may either use alternative receptors for entry into the central nervous system or display altered avidity that permits infection of central nervous system cells which express marginal levels of Bgp receptor (70).

Although Bgp A and Bgp B clearly function as efficient receptors for entry of variant viruses, the efficient MAb CC1 blockade in BHK-MHVR but not in DBT cells argues that these viruses use alternative receptors for entry into DBT cells. Although the identity of the receptor(s) is unclear, variant viruses could interact with an unrecognized polymorphic *Bgp* gene product(s) expressed in DBT cells, such as *Bgp2* (45). Bgp A is a member of the CEA immunoglobulin superfamily, which is composed of a large number of closely related genes (63). MAbs directed against individual CEA proteins usually cross-react with other members in the gene family (52, 57, 63), and sequence analysis demonstrates a high degree of protein identity among different CEA subfamilies, especially within the IgC-like elements. Even greater degrees of homology exist within each subfamily (63). It is plausible that the mutations in variant viruses may not only permit recognition of polymorphic BGP alleles (45) but also permit recognition of closely related CEA family members to promote entry. Although less likely, entirely unrelated proteins might also be recognized as receptors for entry into resistant cells. Additional studies will be needed to clarify whether closely related CEA proteins or other proteins act as receptors for entry of variant viruses into resistant cell clones.

Host resistance and the evolution of virus virulence. Among the four types of persistent viral infections in cell culture (39),

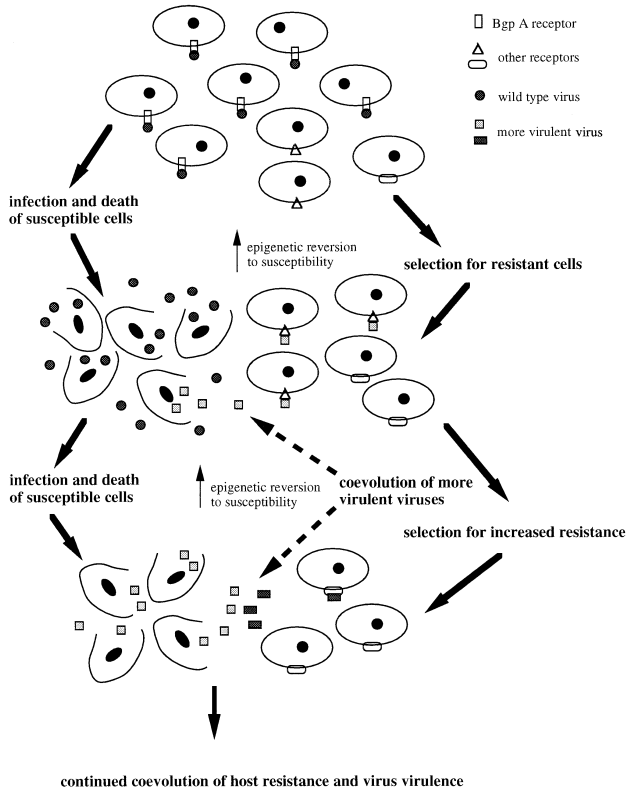

FIG. 9. Mechanism of MHV persistence in vitro. Highly cytolytic MHV infection results in the death of susceptible cell populations, selecting for resistant cells which express little if any BGP receptor. The proliferation of resistant cells forms a genetic bottleneck which selects for the coevolution of more-virulent variant viruses. The variant viruses display increased avidity for BGP receptors and/or recognize new receptors for entry into resistant cells. The emergence of new variant viruses results in additional rounds of selection for new populations of resistant cells and subsequent coevolution of variant viruses, ensuring MHV persistence and the coexistence of the host cells and virus.

carrier cultures are characterized by having a small proportion of the cell population infected with virus. These infected cells lyse, and the progeny virions infect a small number of additional cells. Carrier cultures can be cured with neutralizing antibodies. Persistent infections by MHV as well as most other coronaviruses resemble carrier cultures, since only small percentages of cells are antigen positive and cultures can be cured of virus by the addition of neutralizing antibodies (62). Persistent infections by coronaviruses, like other carrier cultures, are also characterized by inefficient vertical transmission of virus and are dependent upon horizontal transmission for maintenance (14, 15). During persistent infection by MHV-A59 in DBT cells, the inefficient vertical transmission of virus initially resulted from the selection of resistant cells which expressed little if any *Bgp1* proteins, since most of receptor-positive cells were rapidly lysed by acute MHV infection. Resistant *Bgp1*-negative cells proliferated and established an alternative ecological niche which formed a genetic bottleneck for the subsequent selection of more-virulent variant viruses that could proliferate more efficiently in these resistant cells (Fig. 9). By altering their receptor avidities and specificities, these more-virulent variants provided continuous pressure for selecting more-resistant cells in culture. As a result, the persistent infection is maintained not only by epigenetic expression of *Bgp1* and other putative host factors that affect virus replication (69) but also by the coevolution of resistant cells and more-virulent viruses in vitro. Consequently, neither cells nor viruses completely dominate in culture, ensuring dual survival.

Our data predict that extremely virulent viruses which will be incapable of establishing a persistent infection in the original DBT cell line will eventually emerge. Conversely, extremely resistant host cells which cannot be infected by wild-type virus or prevent the establishment of a persistent infection may also emerge. In fact, resistant 17 Cl-1 cells in which parental MHV-A59 could not establish a persistent infection did evolve, yet persistent infections with variant viruses isolated after 1 year postinfection were readily established in these cells (54). Since receptors for transmissible gastroenteritis coronavirus, human coronavirus 229E, bovine coronavirus, and human coronavirus OC43 have been identified and persistent carrier culture infections are readily established in vitro (13, 31, 33, 64, 68), similar coevolutionary mechanisms may also mediate persistence of these coronaviruses in vitro. MHV persistence has been extensively studied with neuronal OBLA21A and primary mixed glial cell cultures (26, 27). Although the mechanisms for persistence in these in vitro models are unknown, initial virus infection is associated with little if any syncytium formation and few cells become infected with virus. The percentage of antigen-positive cells increased with time, and fusion-negative mutants emerged within 2 months postinfection. These viral mutants contain alterations within a highly conserved putative host protease cleavage site and within the fusigenic domain of the S glycoprotein gene (26, 27). Since wild-type MHV-A59 infection in these cell cultures does not elicit cell fusion, as it does in other cell cultures, the coevolutionary pressures likely favored the emergence of mutant viruses which did not require syncytium formation for efficient transmission and replication. Through 119 days postinfection, we have not identified variant viruses with altered fusigenic capacities, although coevolving host ecologies might select such mutants at later times in persistence.

While many theorists have suggested that host-parasite systems coevolve from increased parasitism to commensalism and reduced virulence, others have argued that natural selection may favor increased pathogen virulence in systems in which host pathology clearly favors increased pathogen transmission (35). While some pathogens may become less virulent, many examples point to the contrary proposition of pathogens evolving virulent countermeasures to increasing host resistance. Not surprisingly, evidence suggests that many human viruses have become less virulent than they were in the past, which is attributed primarily to the emergence of host resistance to infection (35). Host resistance to infection is a complex process governed by numerous genes, relatively few of which have been identified. Genes affecting host resistance to malaria, cholera, and tuberculosis infections have been reported (24, 25, 40, 58, 59), and the human *Mx* gene product (MXA) protects cultured cells against infection with influenza virus and vesicular stomatitis virus (48). Major histocompatibility complex polymorphisms clearly contribute to increased resistance or susceptibility to different virus infections (3, 5). In our in vitro model, coevolutionary mechanisms have apparently selected for more-virulent viruses that did not evolve toward commensalism but rather evolved increased virulence as a means of countering increased host cell resistance in vitro. Infection by these highly evolved variant viruses in unselected host cell populations resulted in tremendous lethality and cell death, akin to the devastation caused by smallpox and measles viruses at the organism level following introduction into indigenous, naive New World populations (5). The introduction of highly virulent myxoma viruses into rabbits in Australia resulted in the rapid selection for more-resistant rabbits and the coselection for

attenuated variant viruses which had high levels of transmissibility in a given rabbit population. Also seen was the selection for more-virulent viruses in the rabbit populations that had the highest innate resistance (22). It seems possible that coevolving host resistance and virus virulence over many generations in geographically isolated populations may contribute to the emergence of extremely virulent pathogens following transmission into naive external populations.

Coevolution in a biological system requires an interdependence between the modes of evolution of both entities (20, 35, 36). Coevolution has been hypothesized to occur during reovirus persistence in vitro, in which variant viruses which replicated more efficiently than wild-type viruses in parental and cured persistent cell clones were selected (15). While similar observations have been made for persistently infected cultures of FMDV and lymphotropic minute virus of mice (12, 50), the molecular mechanisms that modulate virus-host coevolution have not been elucidated. We have identified at least two sites of virus-host interaction which coevolve with increasing host cell resistance and likely modulate virus virulence in vitro. The 5′-UTR mutation in some variant viruses increased translation and expression of the MHV polymerase genes in cell culture, thereby enhancing virus gene expression and replication (10). A second mutation site likely involves genetic changes which remodel the S glycoprotein gene so that the variant viruses can interact more efficiently with BGP receptors and/or recognize an alternative receptor(s) for entry, thereby circumventing increased host resistance. These data suggest that MHV persistence may represent a unique biological model to identify the genetic determinants of virus-host interaction that regulate host resistance and virus virulence at the cellular level. This model also may reflect important sites of virus-host coevolution in vivo, since the resistant SJL/J mouse expresses a polymorphic Bgp B receptor that is poorly utilized by MHV-JHM and MHV-A59. MHV-3 however, replicates efficiently and produces disease in the SJL/J mouse (8, 69). The variant viruses isolated in this study will likely allow us to identify high-affinity receptor binding sites which regulate virus entry, tissue tropism, and pathogenesis in cell culture and in animals.

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