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Expression of Cellular Oncogene Bcl-xL Prevents Coronavirus-Induced Cell Death and Converts Acute Infection to Persistent Infection in Progenitor Rat Oligodendrocytes

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Murine coronavirus mouse hepatitis virus (MHV) causes persistent infection of the central nervous system (CNS) in rodents, which has been associated with demyelination. However, the precise mechanism of MHV persistence in the CNS remains elusive. Here we show that the progenitor oligodendrocytes (central glial 4 [CG-4] cells) derived from newborn rat brain were permissive to MHV infection, which resulted in cell death, although viral replication was restricted. Interestingly, treatment with fetal bovine serum or exogenous expression of cellular oncogene Bcl-xL prevented CG-4 cells from MHV-induced cell death. Significantly, overexpression of Bcl-xL alone was sufficient to convert acute to persistent, nonproductive infection in CG-4 cells. This finding indicates that intracellular factors rather than viral components play a critical role in establishing viral persistence in CNS cells. Although viral genomic RNAs continuously persisted in Bcl-xL-expressing CG-4 cells over 10 passages, infectious virus could no longer be isolated beyond 2 passages of the cell. Such a phenomenon resembles the persistent MHV infection in animal CNS. Thus, the establishment of a persistent, nonproductive infection in CG-4 cells may provide a useful in vitro model for studying viral persistence in animal CNS. The data also suggest that direct virus-host cell interaction is one of the underlying mechanisms that regulate viral persistence in CNS cells.

Virus-host cell interaction plays an important role in controlling the outcome of viral infections. Many viruses, including the murine coronavirus mouse hepatitis virus (MHV), can cause lytic infection via necrosis and/or apoptosis in various cell types; however, many types of host cells also have the ability to overcome virus killing (i.e., killing induced by virus). The interplay between a virus and its host cells determines the outcome of an infection. As exemplified by Sindbis virus infection in neuronal cells, overexpression of a single cellular oncogene Bcl-2 can convert lytic to persistent productive infection (28).

Bcl-xL is an antiapoptotic member of the growing Bcl-2 family of cell death regulators. It has a molecular mass of ~25 kDa (5). Like Bcl-2, Bcl-xL is an integral membrane protein that localizes to mitochondrial, endoplasmic reticulum, and nuclear membranes (5, 13). Unlike Bcl-2, which shows a peak in expression during embryonic development and declines postnatally, Bcl-xL expression increases postnatally, reaching a peak in the adult (11, 12, 23). Bcl-xL interacts with a number of the proapoptotic members of the Bcl-2 family and antagonizes their proapoptotic effects. It has been shown that Bcl-xL protects cells from diverse death-inducing stimuli (35). In the central nervous system (CNS), Bcl-xL plays a critical role in protecting neurons from cell death (36). Mice that are gene eliminated for Bcl-xL die around embryonic day 13 and exhibit massive cell death of the developing CNS (36). The abundant expression of Bcl-xL protein in developing and adult brain and the absolute requirement for Bcl-xL during neurogenesis in the mouse suggest that Bcl-xL may be a potent survival factor for the CNS during development and adult tissue homeostasis (35).

MHV is an enveloped RNA virus that contains a single-strand, positive-sense RNA genome. Infection of host cells by MHV is initiated by interaction between virion spike protein and the cell receptors, which then triggers fusion between the viral envelope and the plasma membrane or the endosomal membrane, the latter of which follows receptor-mediated endocytosis (22, 38). The cellular receptors for MHV are members of the carcinoembryonic antigen family of the immunoglobulin superfamily (8, 54, 57). Upon entry, translation of the viral RNA-dependent RNA polymerase initiates from the incoming genomic RNA, and viral replication then proceeds in the cytoplasm of infected cells (24).

MHV infects rodents, the natural hosts, and causes hepatitis, nephritis, enteritis, and CNS diseases. Because the histopathology of the CNS demyelinating disease in rodents induced by some MHV strains, such as JHM, is very similar to those seen in human multiple sclerosis (MS) patients, MHV has been extensively used as an animal model for studying the pathogenesis of MS and other demyelinating diseases. In mice, demyelination can be observed as early as 6 days postinfection (p.i.) and appears to be most extensive at around 4 weeks p.i., at which time infectious virus can no longer be isolated, although viral RNA is still detectable (7, 20, 26). The mechanism of MHV-induced demyelination is not completely understood, although the host immune system has been implicated as mediators of the pathological changes (10, 18, 32, 33, 48, 53, 55, 56). It has been shown that viral persistence in the CNS correlates with demyelination; however, although persistent infection is necessary, it alone is not sufficient for the development of demyelination (7). In addition, apoptotic oligodendrocytes

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have been detected in and near the demyelinating lesions in the CNS of mice and rats infected with MHV strains A59 and JHM (2, 25, 45, 46, 55), suggesting a role for apoptosis in demyelination. In support of the in vivo observation, recent in vitro results have shown that MHV can directly kill cultured rat oligodendrocytes via apoptosis (30).

The predominant cell types in the CNS of MHV-persistently infected animals are glial cells, especially astrocytes and oligodendrocytes (references 26, 41, and 48 and references therein). Little is known about the mechanism of MHV persistence in the CNS and, to date, there is no good in vitro model for MHV persistence. During the course of studying the rat progenitor oligodendrocytes (central glial 4 [CG-4] cells), we unexpectedly observed that a brief pulse of the progenitor cells with fetal bovine serum (FBS) prevented MHV-induced cell death. Further experiments revealed that the cellular survival gene Bcl-xL plays an important role in rescuing the cells from virus killing. Significantly, expression of Bcl-xL alone converted acute to persistent MHV infection in progenitor oligodendrocytes, suggesting that cellular factors play a critical role in establishing MHV persistence in CNS cells. These progenitor cells, having survived viral killing, could be passaged and continued to harbor viral RNAs, although no infectious viruses could be recovered from these cells. This kind of persistent, nonproductive infection in the progenitor oligodendrocyte culture resembles the phenomenon observed in MHV-infected animal CNS and in the brain biopsies of MS patients (37, 47). The similarity of persistent infection in CG-4 cells and in animal CNS may provide a useful in vitro model for studying the mechanism of MHV persistence in the CNS at cellular and molecular levels.

MATERIALS AND METHODS

Cell, virus, and reagents. The CG-4 cell is a permanent, undifferentiated type 2 oligodendrocyte/astrocyte progenitor cell that was originally established during a primary neural cell culture derived from the brain of newborn Sprague-Dawley rat pups (1 to 3 days postnatal) (31). It was kindly provided by Paul Drew (University of Arkansas for Medical Sciences). CG-4 cells were cultured on poly-L-ornithine (0.1 mg/ml)-coated dishes at a seeding density of 100 cells per 1 mm^2 in serum-free Dulbecco minimal essential medium (DMEM) with N1 supplement (5 μg of transferrin/ml, 16.11 μg of putrescine/ml, 6.29 ng of progesterone/ml, 5.14 ng of selenium/ml), biotin (10 ng/ml), insulin (5 μg/ml), and two growth factors, basic fibroblast growth factor 2 at 5 ng/ml and platelet-derived growth factor AA at 10 ng/ml, as well as streptomycin and penicillin. CG-4 cells were passaged every 6 days. Under this condition, CG-4 cell maintains its undifferentiated progenitor phenotype indefinitely. The progenitor cells were used for virus infection and gene expression throughout the present study. Mouse astrocytoma DBT cells (15) were cultured in Eagle minimal essential medium (EMEM) and were used for virus propagation and plaque assay as described previously (30).

MHV strain JHM was obtained from Michael Lai's laboratory. It was derived from JHM (3) after 15 passages of undiluted virus preparations in cell culture. It contains two pentanucleotide repeats at the 3′ end of the leader RNA and expresses the hemagglutinin/esterase gene more abundantly than its parental JHM (3) strain. This culture-adapted virus has been continuously passaged over the years. It causes severe cytopathic effect in DBT cells. It was propagated in DBT cells and was used throughout the present study.

Virus internalization assay. For virus internalization assay, CG-4 cells were grown on 60-mm dishes in the condition medium. ^{3}H-labeled and sucrose gradient-purified viruses were then used to infect CG-4 cells. Virus internalization assay was performed as described previously (52). Briefly, cells were infected with MHV-JHM at a multiplicity of infection (MOI) of 10 at 4°C for 1 h. Unbound virus was then removed by five washes with phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (BSA) and 0.05% Tween 20. Infected cells were incubated at 37°C. At 45 min after a temperature shift, infected cells were washed with cold PBS and incubated with 0.5 mg of proteinase K/ml at 4°C for 45 min to remove the bound but uninternalized virus particles. Cells were collected with a rubber scraper, mixed with an equal volume of a buffer containing 2 mM phenylmethylsulfonyl fluoride and 6% BSA to inactivate proteinase K, and centrifuged for 30 s at 14,000 rpm in a microcentrifuge (Micromax). Cell pellets were washed with EMEM containing 2% BSA. Cells were then resuspended in EMEM and an equal volume of a solution containing 1% sodium dodecyl sulfate (SDS). The radioactivities in the cell lysates were determined by liquid scintillation counting (Beckman 2500T). Parallel experiments were performed on DBT cells, which were used as a positive control. Three replicas per sample were analyzed.

Assay for cell viability. CG-4 cells were infected with MHV or mock infected. At various time points p.i., both detached and adherent cells were collected and stained with trypan blue (Sigma) by mixing 100 μl of cell suspension with an equal volume of 0.4% trypan blue solution prepared in PBS. A total of ca. 100 cells from each sample were counted in a hemocytometer under a microscope. A ratio of dead versus total cell numbers is the percentage of cell death, and the result is expressed as a mean of three independent experiments.

Reverse transcription-PCR (RT-PCR). For detection of Bcl-xL mRNA expression, CG-4 cells were cultured in the condition medium for 24 h. Medium was then removed and the cells were treated with DMEM containing 10% FBS for 10 min or without FBS. The FBS-containing medium was then removed and replaced with the condition medium for the remaining time of the experiments. Mock (DMEM alone)-treated cells were used as a negative control. At every 2 h after serum treatment, cells were harvested and intracellular RNAs were isolated with the TRIzol reagent (Gibco-Pharmacia). The RNAs were reverse transcribed into cDNAs with Moloney murine leukemia virus reverse transcriptase (Promega) by using a random hexamer oligonucleotide primer (Invitrogen, Inc.) in a standard RT reaction as described previously (58). cDNAs were then used as templates for PCR amplification with two pairs of primers specific for Bcl-xL gene and β-actin gene (as an internal control), respectively. The sense primer for Bcl-xL (5′-TTT *GAA TCC* GCC ACC ATG TCT CAG AGC AAC CGG GAG CTG-3′) corresponds to a sequence at the 5′-end of the open reading frame (ORF) and the antisense primer (5′-TTT *CTC GAG* CTT TCC GAC TGA AGA GTG AGC CCA-3′) contains a sequence at the 3′ end of the Bcl-xL ORF. Both primers also contain a restriction enzyme site XhoI and EcoRI (in italics), respectively, for cloning (see below). The primer pair for β-actin is 5′mb-actin (5′-ACC AAC TGG GAC GAT ATG GAG AAG A-3′, corresponding to the sequence at nucleotides 229 to 253 of the β-actin gene) and 3′mb-actin (5′-TAC GAC CAG AGG CAT ACA GGG ACA-3′, complementary to a sequence at nucleotides 418 to 442 of the β-actin gene; GenBank accession number NM007393). The PCR product is ~700 bp in length for Bcl-xL gene and 215 bp for the β-actin gene. PCR was carried out in a reaction of 20 μl containing 1× PCR buffer (20 mM Tris.HCl [pH 8.3], 25 mM KCl, 1.5 mM $MgCl_2$, 0.1% Tween 20), 200 μM concentrations of each of four deoxynucleoside triphosphates, 20 pmol of primers, 3 U of *Taq* DNA polymerase, and 2 μl of the RT products for 30 cycles, with each cycle consisting of denaturation at 95°C for 30 s, annealing at 55°C for 1 min, and extension at 72°C for 1 min, with a final extension of 10 min. PCR was performed in a thermocycler DNA Engine (model PTC-200; MJ Research). PCR products were analyzed by 1% agarose gel electrophoresis and visualized by staining with ethidium bromide and photographed with a gel document system (UVP).

For detection of MHV N gene in persistently infected cells, the same RT-PCR method was used, with the exception of the primer pair. The primer pair for the N gene was 5′BamN (5′-TAG *GGA TCC* ATG TCT TTT GTT CCT-3′) and 3′EcoN515 (5′-TAG *GAA TTC* GGC AGA GGT CCT AG-3′). The PCR product was 533 bp in length.

Western blot. For detection of cellular Bcl-xL protein, CG-4 cells were either treated with 10% FBS for 10 min or mock-treated as a control. At various time points after serum treatment, cells were lysed with the radioimmunoprecipitation assay buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.5% sodium deoxycholate, 1% NP-40) containing protease inhibitor cocktail tablets (Roche, Mannheim, Germany). The cell lysates were passed through a 25-gauge needle several times to sheer the DNA and were clarified from cell debris by centrifugation. In some cases, the protein concentration was measured by using Bio-Rad protein assay kit (Bio-Rad, Richmond, Calif.). Cellular proteins with equivalent number of cells for each sample were resuspended in 20 μl of Laemmli electrophoresis sample buffer (10 mM Tris-HCl [pH 6.8], 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol), boiled for 3 min, and resolved by SDS-polyacrylamide gel electrophoresis (PAGE) on 12.5% gel. Proteins were then transferred to nitrocellulose membrane (MSI, Westborough, Mass.) overnight at 50 V in a transfer buffer (25 mM Tris, 200 mM glycine, 20% methanol, 0.02% SDS). After being blocked with 5% skim milk in PBS for 1 h at room temper-

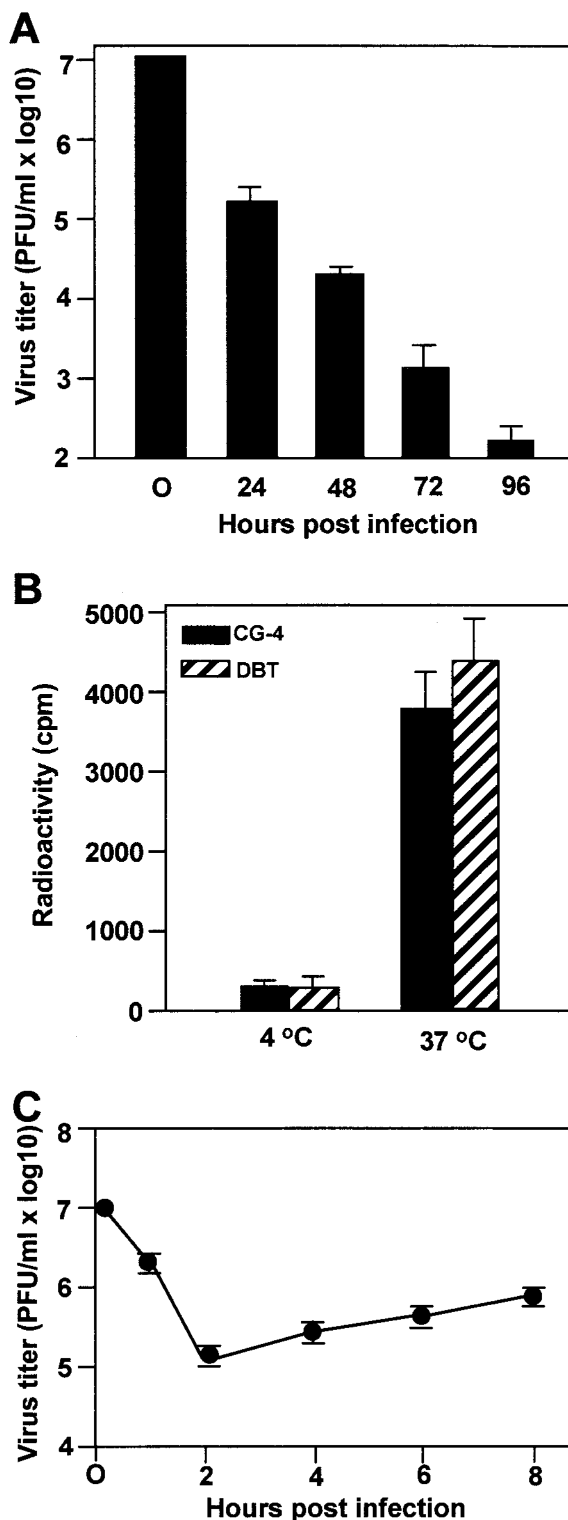

FIG. 1. Internalization and replication of MHV in progenitor rat oligodendrocyte CG-4 cells. (A) CG-4 progenitor cells were grown in condition medium. Approximately 10^6 cells were infected with MHV strain JHM at an MOI of 10. At each time point p.i. as indicated, virus titers recovered from the culture were determined by plaque assay in DBT cells. The results are expressed as the mean PFU per milliliter for three independent experiments. Error bars indicate standard deviations of the means. The virus titer at 0 h p.i. denotes the virus titer for the inoculum. (B) MHV strain JHM was grown in DBT cells and the virus genomic RNAs were labeled with [^{3}H]uridine as described in Materials and Methods. Radiolabeled viruses were then purified by sucrose gradient ultracentrifugation and were used for infection of CG-4 cells. Infection of DBT cells permissive for MHV infection was used as a positive control. Virus attachment was carried out at 4°C for 1 h. After extensive washing of unbound viruses, one set of the cell cultures was moved to 37°C for an additional hour to allow internalization. The other set of cultures remained at 4°C for an additional hour. At the end of the second hour, bound but uninternalized viruses were removed by treatment with protease K. Cells were then lysed, and intracellular radioactivities were determined in a liquid scintillation counter. The results are expressed as the mean counts per minute (cpm) for three independent experiments. Error bars indicate the standard deviations of the means. (C) One step-growth curve in progenitor CG-4 cells. The results are expressed as the mean PFU per milliliter for three independent experiments. Error bars indicate the standard deviations of the means.

ature, nitrocellulose membrane was washed three times in Tris-buffered saline containing 0.1% Tween 20 and immunoblotted with a polyclonal rabbit antibody specific to Bcl-xL (0.2 μg/ml; Cell Signals, Inc.) or a monoclonal antibody specific to β-actin as an internal control (1:2,000; Sigma) for 1 h at room temperature, followed by a secondary goat anti-rabbit immunoglobulin G antibody or anti-mouse immunoglobulin G antibody, respectively, coupled to horseradish peroxidase (1:1,500; Sigma) for 1 h at room temperature. The presence of Bcl-xL and β-actin proteins was detected by enhanced chemiluminescence (ECL) with peracid as a substrate (Amersham Pharmacia Biotech), followed by autoradiography, with exposure times ranging from 30 s to 1 min. For detection of MHV N protein, the primary monoclonal antibody (J3.3.1) specific to MHV N protein was kindly provided by Stephen Stohlman (Keck School of Medicine, University of Southern California, Los Angeles) and was used in the immunoblot.

DNA transfection and selection of stable transfectants. The plasmid containing the Bcl-xL ORF was a gift from Tim Chambers (Department of Biochemistry and Molecular Biology, University of Arkansas for Medical Sciences). CG-4 cells were grown in 60-mm culture plates (Sarsted) to ~60% confluence and were transfected with plasmid DNA pcDNA3/Bcl-xL or pcDNA3 vector by use of the cationic liposome transfection reagent DOTAP according to the manufacturer's instructions (Boehringer Mannheim). Briefly, 5 μg of each construct was mixed with 30 μl of DOTAP in 70 μl of 20 mM HEPES (pH 7.4). The mixture was then added to CG-4 cells. After incubation for 24 h, Geneticin (G418; Gibco-BRL) was added to the medium at the final concentration of 400 μg/ml, and the cells were grown for 2 to 3 days. Cells that died off during this period were removed, whereas surviving cells were allowed to grow further in the presence of G418 replenished with fresh medium every 3 days. Cells that were resistant to G418 treatment for 4 weeks were screened for expression of the Bcl-xL.

RESULTS

MHV infection induced death of progenitor rat oligodendrocyte CG-4 cells. CG-4 cells were chosen for the present study because they not only resemble the primary progenitor oligodendrocytes morphologically, biochemically, and biologically but also can be continuously cultured in vitro under defined conditions even though they are not transformed or immortalized (31, 34). We have shown previously that, in the continuous presence of both growth factors—basic fibroblast growth factor and platelet-derived growth factor—in the condition medium, CG-4 cells maintained their undifferentiated progenitor morphology, i.e., the majority of the cells contained bipolar processes with a few cells having short and relatively unbranched multipolar processes (30). These cells were stained with an antibody specific to A2B5, a molecular marker for progenitor cells (30), thus establishing the progenitor phenotype of the CG-4 cells.

To determine the susceptibility of the progenitor cells to MHV infection, CG-4 cells were infected with the neuroviru-

lent MHV strain JHM at an MOI of 10. At various time points p.i., virus titers in the cell culture were determined by plaque assay. It was found that the virus titers did not increase from days 1 to 4 p.i., indicating that MHV replication is restricted in CG-4 cells (Fig. 1A). To determine whether the restriction resides during cell entry or postentry, we performed virus internalization assay. Viral genomic RNAs were labeled with [^{3}H]uridine, and radiolabeled viruses were used for infection at 4 or 37°C. We found that MHV indeed entered CG-4 cells when the temperature was shifted from 4 to 37°C, as evidenced by the high level of radioactivity (Fig. 1B). The radioactivity was similar to that of DBT cells infected with the same MHV. In contrast, no radioactivity was detected when virus infection remained at 4°C. Taken together, these results confirmed that CG-4 cells are susceptible to MHV infection but that virus replication is nonproductive and restricted in CG-4 cells. To further confirm that the virus truly replicates in CG-4 cells after entry, we determined the virus one-step growth curve. As shown in Fig. 1C, MHV did grow after the eclipse period, albeit inefficiently, thus confirming the restrictive nature of MHV replication in CG-4 cells.

To determine the effect of MHV infection on CNS cells, CG-4 cells were infected with MHV strain JHM at an MOI of 10 to ensure that every cell was infected. The cytopathic effect was then observed daily for 4 days p.i. under a microscope. In parallel, the cell viability was determined with trypan blue staining. As shown in Fig. 2, infected cells began to become rounded and clumped together at 24 h p.i. These cells began to detach from the culture dishes at 48 h p.i. and almost completely detached from the dishes by 4 days p.i. (data not shown). Occasionally, syncytia could be observed (Fig. 2A). This result reveals the cytopathic effect of MHV in CG-4 cells. To corroborate this observation and to quantitatively determine the cytopathic effect, we performed trypan blue staining. At 24 h p.i., ca. 10% more cells died in virus-infected cells compared to those of mock-infected cells (Fig. 2B). The number of dead cells increased continuously and significantly from days 1 to 4 p.i. (from 20 to 50%) in virus-infected culture, whereas it remained at a similar level during the same period of time in mock-infected cells(ranging from ca. 10 to 20%). Although a substantial fraction (>40%) of the infected cells were viable by trypan blue staining at day 4 p.i., these cells were detached from the dishes and could no longer be passaged. These results demonstrate that CG-4 cells could be killed by MHV infection even though virus replication was restricted and nonproductive.

Brief treatment with serum prevented CG-4 cells from virus killing. During the course of the present study, we unexpectedly observed that CG-4 cells that were briefly pulsed with serum could resist virus killing. Initially, when we subcultured the virus-infected CG-4 cells at 12 h p.i. and treated the cells with 10% FBS for 10 min, we found that these infected cells survived virus infection and could be continuously passaged numerous times. This observation suggests two possible scenarios: the serum or the subculturing process stimulates cell growth. To determine which of the two contributes to the resistance, CG-4 cells were infected with MHV-JHM. At 12 h p.i., cells were either treated with 10% serum for 10 min or untreated. Fresh medium was then added to both cell cultures. We found that serum-treated, MHV-infected cells grew well during the first 3 days p.i. (similar to mock-infected cells), whereas a significantly larger percentage of the untreated, MHV-infected cells died during the same period (Fig. 3). In contrast, subculturing of cells at 12 h p.i. without serum treat-

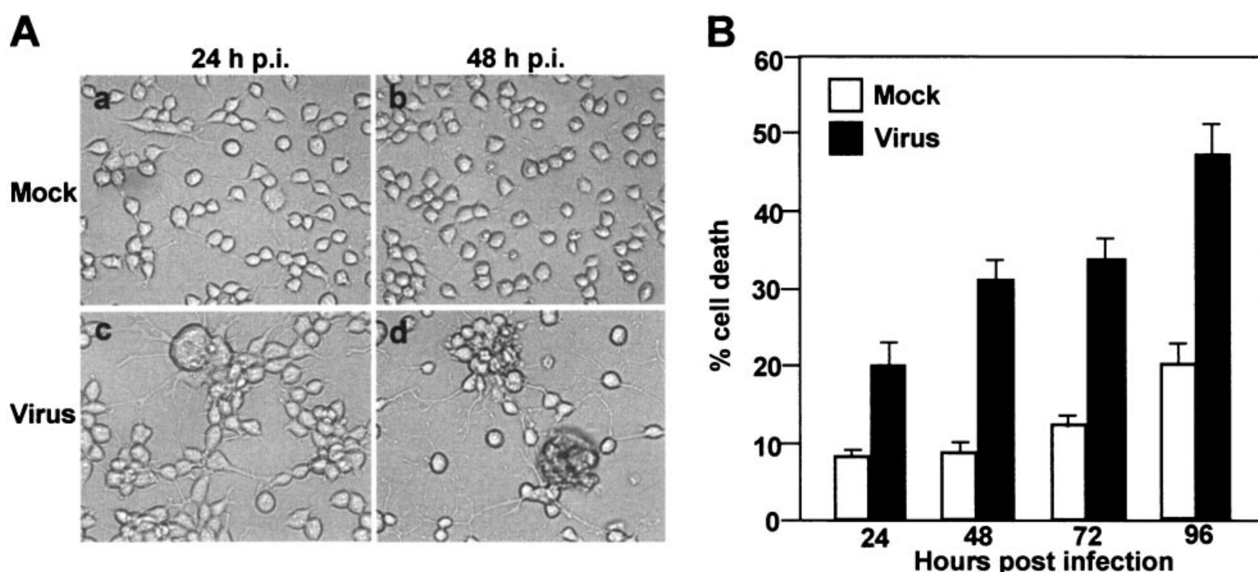

FIG. 2. MHV induces cell death in progenitor rat oligodendrocyte CG-4 cells. Progenitor CG-4 cells were mock infected (Mock) or infected with MHV-JHM at an MOI of 10 (Virus). (A) At the indicated time points postinfection (hours p.i.), a set of the cells were examined for cytopathic effect under a microscope. All photographs were taken with the digital camera (MagnaFire) attached to the microscope (Olympus IX70) with the same magnification. (B) The other sets of cells were collected and stained with trypan blue and analyzed for cell viability. The numbers of both live and dead cells were counted in a hemocytometer under a microscope. A total of ca. 100 cells were counted for each sample. The bar in each graph denotes the mean percentage of cell death for at least three independent experiments. Error bars indicate the standard deviations of the means.

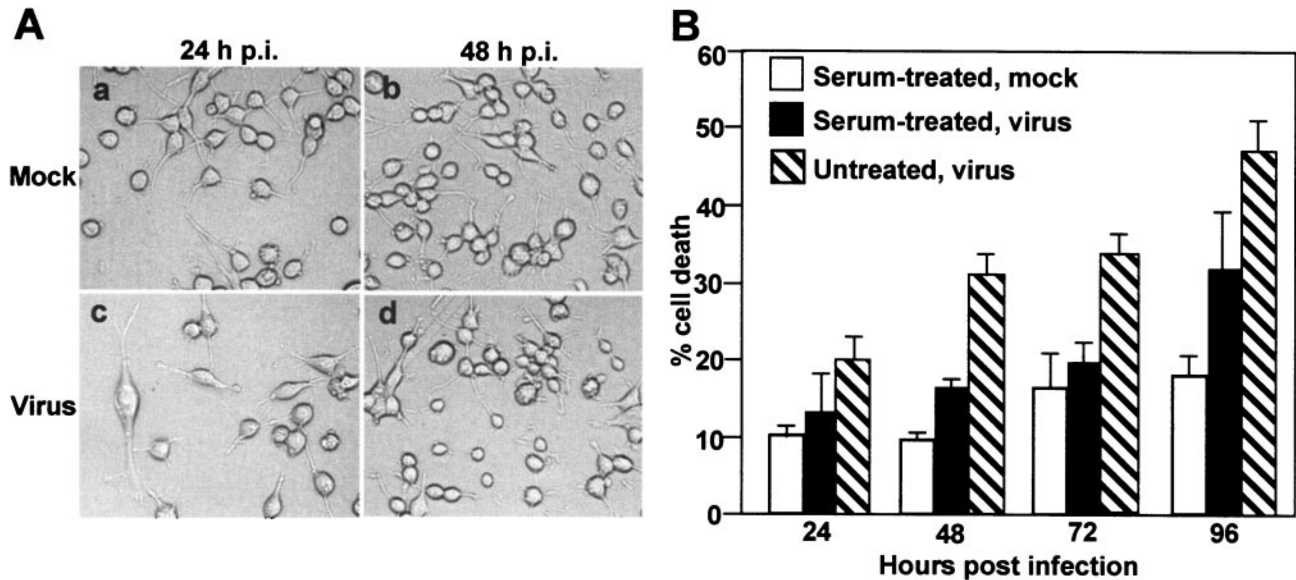

FIG. 3. Treatment of CG-4 cells with serum prevents MHV-induced cell death. Progenitor CG-4 cells were mock infected (Mock) or infected with MHV-JHM at an MOI of 10 (Virus). At 12 h p.i., cells were treated with 10% FBS in the condition medium for 10 min (Serum-treated). Serum-containing medium was then removed and replaced with the same condition medium without serum. (A) At the indicated time points p.i., a set of the cells were examined for cytopathic effect under a microscope. All photographs were taken with the digital camera (MagnaFire) attached to the microscope (Olympus IX70) with the same magnification. (B) The other sets of cells were collected and stained with trypan blue and were analyzed for cell viability. Cells that were infected with the virus but untreated with serum (Untreated) were used as a control for direct comparison. The numbers of both live and dead cells were counted in a hemocytometer under a microscope. A total of ca. 100 cells were counted for each sample. The bar in each graph denotes the mean percentage of cell death for at least three independent experiments. Error bars indicate the standard deviations of the means.

ment did not prevent cell death (data not shown). These results confirm that the serum treatment rather than the subculturing process stimulated cell growth and protected cells from virus killing.

Induction of Bcl-xL expression in CG-4 cells by serum treatment. Because serum contains a large number of growth factors or mitogens, it is likely that these mitogens stimulate cellular survival gene expression and promote cell growth. To test this hypothesis, we determined the gene expression level of a cellular survival gene Bcl-xL as an example. CG-4 cells were either treated with 10% FBS for 10 min or mock treated. At various time points posttreatment, intracellular RNAs and proteins were isolated. The level of Bcl-xL mRNA was determined by RT-PCR with β-actin mRNA as an internal control. As shown in Fig. 4A, Bcl-xL mRNAs increased continuously from 2 to 8 h and peaked at 8 h after serum treatment. In contrast, no increase in Bcl-xL mRNA was detected in mock-infected cells. It is noted that because this was not real-time, quantitative PCR, slight variations among individual samples are expected. The level of Bcl-xL protein was then determined by Western blot analysis. Consistent with the level of the mRNA, Bcl-xL protein level also increased after serum treatment when normalized with β-actin protein (Fig. 4B). These results clearly indicate that Bcl-xL gene expression was induced in CG-4 cells by brief serum treatment.

Overexpression of Bcl-xL alone is sufficient to rescue CG-4 cells from virus killing. The results presented above suggest that Bcl-xL might be one of the cellular mitogen-activated genes that contribute to the survival of CG-4 cells after MHV infection. To provide direct evidence for the role of Bcl-xL in protecting CG-4 cells from virus killing, we cloned the Bcl-xL gene into a eukaryotic expression vector under the control of a cytomegalovirus immediate-early gene promoter. Cells were transfected with pcDNA3/BclxL DNA, and stable expressing cells were selected by G418. Expression of Bcl-xL was detected by Western blotting with an antibody specific to Bcl-xL (Fig. 5A). It should be noted that due to the special growth requirement for primary glial cell culture, it was not possible to clone individual cells. Therefore, the stable cell cultures are mixed populations representing cells with various levels of Bcl-xL expression. Nevertheless, empty-vector-transfected cells as a negative control expressed little or no Bcl-xL, even with overexposure of the gel (Fig. 5A, lane pcDNA3).

Next, CG-4 cells stably expressing Bcl-xL or the vector alone were infected with MHV-JHM at an MOI of 10. At various time points postinfection, the cytopathic effect and cell viability were determined. As shown in Fig. 5B and C, fewer Bcl-xL-expressing cells died after virus infection (7.3 to 22.5% dead cells from days 1 to 4 p.i.) compared to vector-transfected cells (20 to 50% dead cells from days 1 to 4 p.i.). These results thus provide direct evidence that overexpression of cellular oncogene Bcl-xL alone could prevent CG-4 cells from being killed by virus.

Conversion of acute to persistent MHV infection in CG-4 cells by overexpression of Bcl-xL. Since the expression of Bcl-xL could overcome virus killing, an immediate question was whether the surviving cells still harbored the virus for a prolonged period of time. To address this question, CG-4 cells stably expressing Bcl-xL were infected with MHV-JHM at an MOI of 10 and continuously cultured for 4 days in the presence

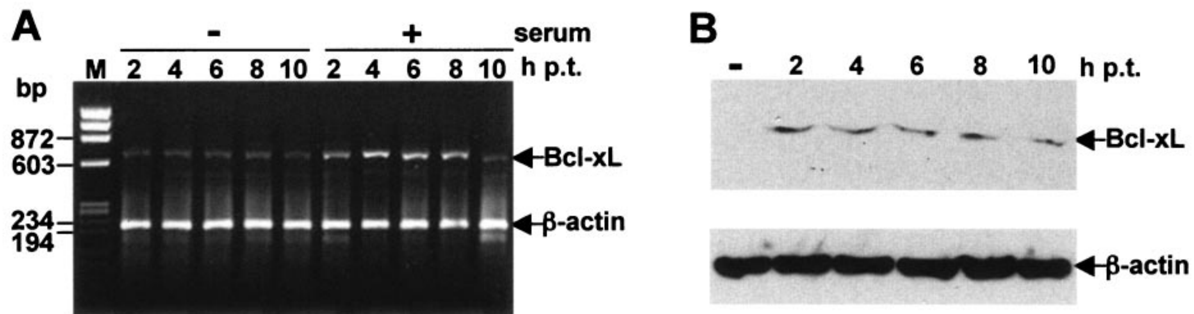

FIG. 4. Induction of cellular oncogene Bcl-xL expression by serum treatment. CG-4 cells were grown in 60-mm dishes in condition medium overnight and were treated with 10% FBS for 10 min. Serum was removed and replaced with the same condition medium. At various time points (in hours) after serum treatment as indicated (h p.t.), cells were collected for analyzing the expression of Bcl-xL gene by RT-PCR (A) and Western blotting (B). (A) RT-PCR analysis of Bcl-xL mRNA. Intracellular RNAs were isolated with the TRIzol Reagent (Gibco-Pharmacia). cDNAs were synthesized with the random hexamer primer by RT. Bcl-xL gene and the control β-actin gene were amplified in the same PCR with the gene-specific primer pairs as described in Materials and Methods. RT-PCR products were analyzed by 1% agarose gel electrophoresis. The gene-specific bands are indicated by the arrows on the right. −, No serum treatment; +, with serum treatment. Molecular mass markers (M) in base pairs (bp) are shown on the left. (B) Western blot analysis of Bcl-xL protein. Cellular proteins were extracted with the cell lysis buffer, separated by PAGE (12.5% gel), transferred to nitrocellulose membrane, and detected with antibodies specific to Bcl-xL and β-actin as described in Materials and Methods. Arrows on the right indicate the specific protein bands. −, No serum treatment. The data are representative of at least three independent experiments.

of G418. Cells were then passaged regularly. At various passages, cells were harvested. The presence of the viral N gene was determined by RT-PCR with N gene-specific primers, and the presence of the N protein was determined by Western blot analysis with an N-specific monoclonal antibody. As shown in Fig. 6A, N gene was detected in all 10 passages, although the level decreased slightly in passages 8 and 10. To further determine whether the viral RNA is truly persistent, we passaged the cells for an additional 10 passages. We found that the N gene was still present in passage 20 (p20) (Fig. 6A, right panel), confirming the persistent nature of virus infection. The identity of the N gene PCR products was confirmed by DNA sequencing (data not shown). The N protein was also detected in the first eight passages but was undetectable in further passages by Western blotting (Fig. 6B). Immunofluorescence staining to the N protein was detected in most of the cells at P0, ca. 30 and 10% of the cells at P1 and P2, respectively, and was undetectable at P3 (data not shown). However, infectious virus could no longer be isolated from the cells beyond passage 2 (Fig. 6C). We then attempted to recover infectious virus by using two alternative approaches: one was to coculture DBT cells with the persistently infected CG-4 cells (passage 10), and the other was to induce fusion between DBT cells and CG-4 cells by polyethylene glycol. Both approaches failed to recover any infectious virus (data not shown). These results demonstrate that Bcl-xL-expressing CG-4 cells recovered from virus infection continuously harbored the viral RNA but not the infectious virus, suggesting that Bcl-xL has the ability to convert acute infection to persistent, nonproductive infection in CG-4 cells, a phenomenon observed in animal CNS.

DISCUSSION

In the present study, we have shown that MHV enters progenitor oligodendrocyte CG-4 cells but that virus replication appears restricted and nonproductive, as evidenced by a decrease in virus titer from days 1 to 4 p.i. (Fig. 1). A similar phenomenon has been observed previously in mature oligodendrocytes differentiated in vitro from progenitor CG-4 cells (30). It has been previously suggested that the restriction of mature oligodendrocytes to MHV infection occurs at the uncoating stage after viral entry. Because uncoating requires dephosphorylation of the N protein by certain cellular phosphatases, increased levels of kinases in mature oligodendrocytes block dephosphorylation of N protein, thereby inhibiting virus uncoating (19, 39). Whether a similar phenomenon exists in progenitor CG-4 cells and whether cellular factors other than the kinases contribute to the inhibitory effects on virus production remain to be investigated.

Despite the fact that MHV replication is restricted in progenitor CG-4 cells, MHV infection results in cytopathic effect and ultimately in cell death (Fig. 2). Although some of the infected cells did not die within the observation period (4 days p.i.), these cells could no longer be subcultured and died eventually (data not shown). When the cellular DNAs were stained with propidium iodide and observed under a microscope, we found that both necrosis and apoptosis are involved in the cell death (data not shown). In a previous study, we demonstrated that MHV infection induces apoptosis in CG-4-derived oligodendrocytes. However, whether necrosis is also involved in oligodendrocyte death has not been ruled out (30). It should be noted that the type and mechanism of CG-4 cell death induced by MHV infection are not the focus of the present study but are interesting issues to be addressed in the future.

Significantly, overexpression of a single cellular survival gene can convert acute infection to persistent, nonproductive infection (Fig. 6). A similar instance has been observed in Sindbis virus-infected neuronal or fibroblast cells, in which overexpression of the cellular survival gene Bcl-2 alone can prevent cell death and convert lytic to persistent infection (28). However, unlike Sindbis virus persistence in these cells, MHV persistence in CG-4 cells is nonproductive. No infectious virus has been recovered once the persistently infected cells are passed beyond two passages. Interestingly, viral genomic RNA se-

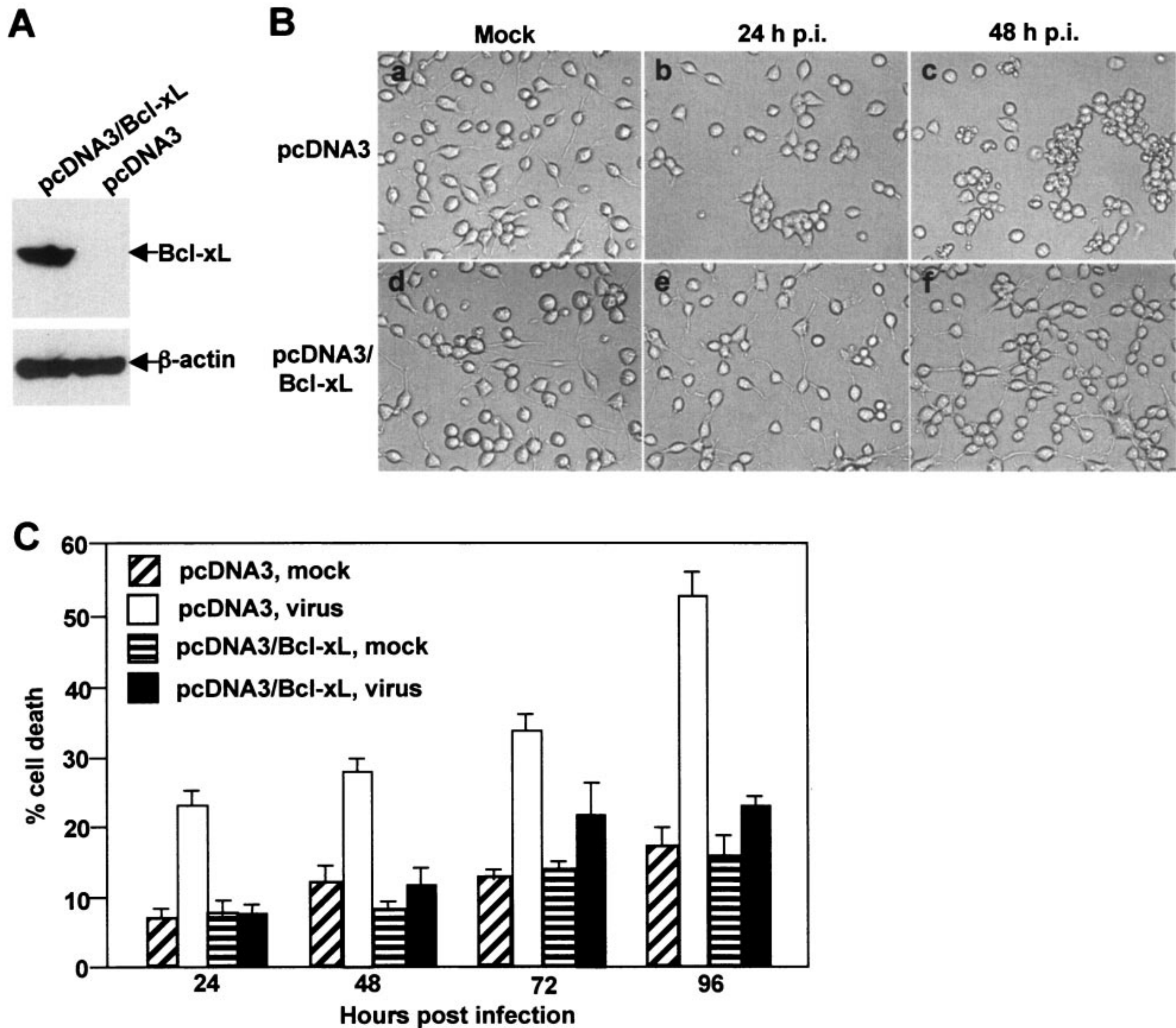

FIG. 5. Overexpression of cellular oncogene Bcl-xL alone is sufficient to prevent CG-4 cells from virus killing. (A) Progenitor CG-4 cells were transfected with pcDNA3/Bcl-xL or the empty vector pcDNA3 and were selected by G418. G418-resistant cells were analyzed for stable expression of Bcl-xL by Western blotting. Cellular proteins were extracted with the cell lysis buffer, separated by PAGE (12.5% gel), transferred to nitrocellulose membrane, and detected with a Bcl-xL-specific antibody and an enhanced chemiluminescence system as described in Materials and Methods. β-Actin was used as an internal control for protein loading. (B) CG-4 cells stably expressing Bcl-xL (CG-4/Bcl-xL) or vector alone (CG-4/pcDNA3) were mock infected (Mock) or infected with MHV-JHM at an MOI of 10 (Virus). At the indicated time points p.i., a set of the cells were examined for cytopathic effect under a microscope. All photographs were taken with the digital camera (MagnaFire) attached to the microscope (Olympus IX70) with the same magnification. (C) The other sets of cells were collected, stained with trypan blue, and analyzed for cell viability. The numbers of both live and dead cells were counted in a hemocytometer under a microscope. A total of ca. 100 cells was counted for each sample. The bar in each graph denotes the mean percentage of dead cells for at least three independent experiments. Error bars indicate the standard deviations of the means.

quences have been consistently amplified from the persistently infected cells. These characteristics of persistence in CG-4 cells closely resemble those observed in the persistently MHV-infected CNS of mice (26, 40). Previous attempts to establish persistent MHV infection in cell cultures, including the mouse astrocytoma cell line DBT and fibroblast cell lines, have been successful (6, 44), and infectious viruses have been produced in these persistently infected cells continuously passaged over 3 years (44). Persistent productive infection has also been established in primary mouse glial cell culture (27). However, such a persistent, productive infection in cell culture is fundamentally different from that seen in animal CNS. Thus, the establishment of a persistent, nonproductive infection in progenitor oligodendrocyte CG-4 cells may provide a useful in vitro model for studying MHV persistence in CNS cells.

MHV persistence in rodent CNS is associated with demyelinating encephalomyelitis. Recent studies have established that MHV persistence is required for CNS demyelination but that persistence alone is not sufficient to cause demyelination in mice (7). The precise mechanisms of MHV persistence in the

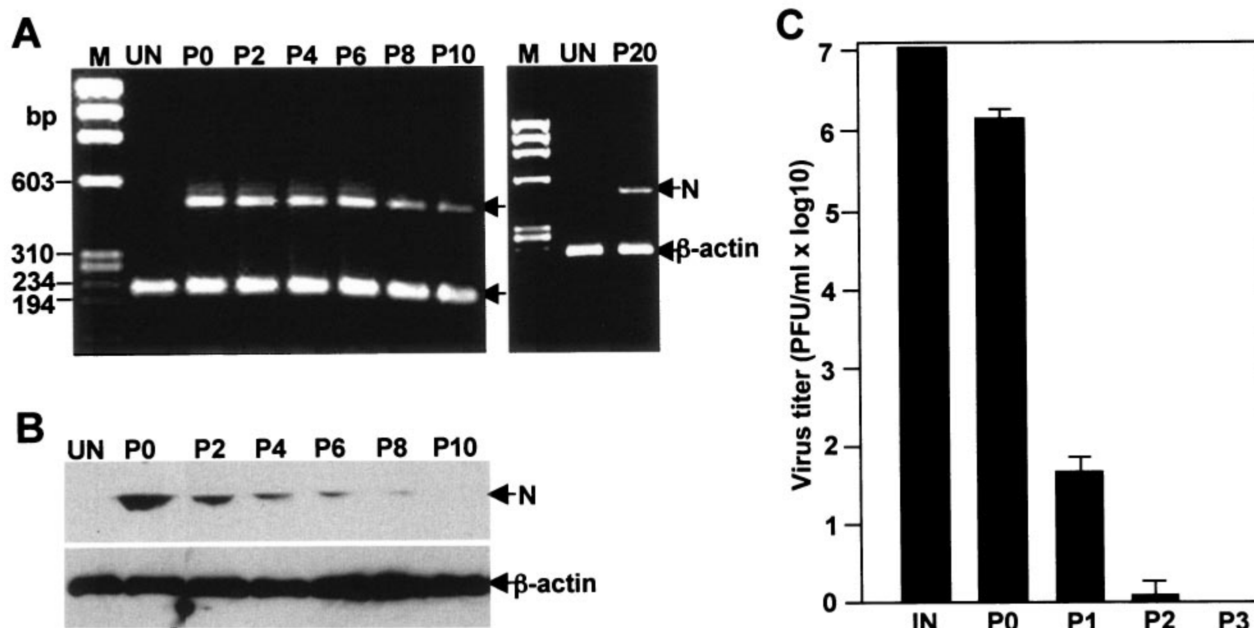

FIG. 6. Overexpression of cellular oncogene Bcl-xL in CG-4 cells converts acute infection to persistent, nonproductive infection. CG-4 cells stably expressing Bcl-xL (CG-4/Bcl-xL) were either mock infected (UN) or infected with MHV at an MOI of 10. Cells that were initially infected with the virus are designated passage 0 (P0). Cells were then passaged every 3 days. The resultant passages are termed P1, P2, and so forth. Cells from each passage were analyzed for the presence of the viral N gene by RT-PCR (A), viral N protein by Western blotting (B), and infectious virus by plaque assay (C). (A) RT-PCR detection of viral N gene. After the extraction of total intracellular RNAs with the TRIzol reagent, cDNAs were synthesized with the random hexamer primer by RT. The N gene and the control β-actin gene were amplified in the same PCR with gene-specific primer pairs as described in Materials and Methods. RT-PCR products were analyzed by 1% agarose gel electrophoresis. Molecular size markers (M) in base pairs (bp) are shown on the left. The upper and lower bands in both panels are the N gene and β-actin gene, respectively, as indicated by arrows. (B) Western blot detection of viral N protein. Cellular proteins were extracted with the lysis buffer, separated by PAGE (10% gel), transferred to nitrocellulose membrane, and detected with antibodies specific to MHV N protein, and β-actin as described in Materials and Methods. Arrows on the right indicate the specific protein bands. (C) Virus recovery from various cell passages. IN, input virus titer for primary infection. The results are expressed as the mean PFU per ml for three independent experiments. Error bars indicate the standard deviations of the means.

CNS cells are not well understood, although several hypotheses have been proposed (4, 9, 17, 21, 49). One hypothesis suggests that downregulation of viral gene expression and function is an important mechanism for viral persistence in cell cultures (4, 17). Leader RNA heterogeneity and mutations in the leader during persistent infection give rise to an intraleader ORF, which attenuates translation of downstream ORFs of bovine coronavirus (17). Cultures persistently infected by MHV also generate defective-interfering RNAs and various types of mutants (14, 16, 21, 49–51). However, it is not clear whether these genomic alterations are the consequence or the cause of viral persistence. Fleming and coworkers systematically analyzed regions of MHV genome isolated from mouse brains at various days (4 to 100 days) p.i. and showed that significant mutations and quasispecies of the viral RNAs accumulated during persistence, suggesting that mutations are likely the result rather than the cause of persistence (1, 43). Other studies suggest that downregulation of MHV receptor expression on host cells is an important mechanism for MHV persistence in culture (44). Persistent MHV infection is readily established in astrocytoma cell line DBT and fibroblast cell line 17Cl-1 (3, 6, 44). Both cell lines are highly heterogeneous in expression level of the MHV receptor, which correlates well with their susceptibility to MHV infection. Thus, in mixed populations of cells, the epigenetic expression of MHV receptor allows the establishment of a persistent infection (44). This suggests that persistent MHV infection may not be established in cell populations that express high levels of MHV receptors. In the present study, we have shown that expression of a single cellular oncogene can rescue progenitor oligodendrocytes, which are highly susceptible to MHV infection, from being killed by virus and can convert acute infection to persistent, nonproductive infection (Fig. 6). This finding suggests that general intracellular molecules play a critical role in establishing viral persistence in glial cells. Although the mechanism by which intracellular genes contribute to the establishment of MHV persistence remains to be determined, based on the findings described here, we propose the following model. Acute infection of oligodendrocytes results in cytopathic effect. However, once the oligodendrocytes overcome acute virus killing, they become persistently infected, partly due to the restrictive nature of the oligodendrocytes to MHV replication. Cellular survival genes such as Bcl-xL indirectly facilitate the establishment of persistent MHV infection by preventing infected cells from virus killing. Therefore, the role of Bcl-xL in MHV persistence is rather indirect.

Stohlman and coworkers have shown that neutralizing antibodies play a critical role in establishing and maintaining per-

sistent MHV infection in the mouse CNS and cell culture (29, 42). Neutralizing antibodies to the spike protein were transferred into MHV-infected B-cell-deficient mice after initial virus clearance and suppressed virus recrudescence, which was associated with reduced demyelination (42). In MHV-infected DBT cells, persistent infection was maintained in the presence of neutralizing antibodies to the viral spike protein (42). Removal of antibodies from the medium restored virus titer and cell fusion (42). A possible interpretation is that the antibody neutralizes the released extracellular virus, thus preventing reinfection of uninfected cells. The neutralizing antibodies can also interact with the spike proteins expressed on the surface of infected cells, thereby preventing fusion with neighboring cells, whereas the intracellular virus replication is not severely blocked. In the present study, no neutralizing antibodies were added to the culture medium, and little or no infectious viruses were produced in persistently infected CG-4 cells, so maintenance of the viral genomic RNAs is likely controlled by intracellular factors. Our finding that expression of a cellular protein Bcl-xL alters the course of MHV infection from acute to persistent, nonproductive infection in CNS cells is consistent with this interpretation. It also suggests that MHV persistence in the CNS cells is the consequence of a complex interplay between the virus and the host. Future research will be directed to determine what cellular factors control MHV replication and persistence in CG-4 cells and other glial cells.

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REFERENCES

1. **Adami, C., J. Pooley, J. Glomb, E. Stecker, F. Fazal, J. O. Fleming, and S. C. Baker.** 1996. Evolution of mouse hepatitis virus (MHV) during chronic infection: quasispecies nature of the persisting MHV RNA. Virology **209:** 337–346.
2. **Barac-Latas, V., G. Suchanek, H. Breitschopf, A. Stuehler, H. Wege, and H. Lassmann.** 1997. Patterns of oligodendrocytes pathology in coronavirus-induced subacute demyelination encephalomyelitis in the Lewis rat. Glia **19:**1–12.
3. **Baric, R. S., E. Sullivan, L. Hensley, B. Yount, and W. Chen.** 1999. Persistent infection promotes cross-species transmissibility of mouse hepatitis virus. J. Virol. **73:**638–649.
4. **Baybutt, H. N., H. Wege, M. J. Carter, and V. Ter Meulen.** 1984. Adaptation of coronavirus JHM to persistent infection of mouse Sac(−) cells. J. Gen. Virol. **65:**915–924.
5. **Boise, L. H., M. Gonzalez-Garcia, C. E. Postema, L. Ding, T. Lindsten, L. A. Turka, X. Mao, G. Nunez, and C. B. Thompson.** 1993. bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. Cell **74:**597–608.
6. **Chen, W., and R. S. Baric.** 1996. Molecular anatomy of mouse hepatitis virus persistence: coevolution of increased host cell resistance and virus virulence. J. Virol. **70:**3947–3960.
7. **Das Sarma, J., L. Fu, J. C. Tsai, S. R. Weiss, and E. Lavi.** 2000. Demyelination determinants map to the spike glycoprotein gene of coronavirus mouse hepatitis virus. J. Virol. **74:**9206–9213.
8. **Dveksler, G. S., M. N. Pensiero, C. B. Cardellichio, R. K. Williams, G. Jiang, K. V. Holmes, and C. W. Dieffenbach.** 1991. Cloning of the mouse hepatitis virus (MHV) receptor: expression in human and hamster cell lines confers susceptibility to MHV. J. Virol. **65:**6881–6891.
9. **Fleming, J. O., J. J. Houtman, H. Alaca, H. C. Hinze, D. McKenzie, J. Aiken, T. Bleasdale, and S. Baker.** 1994. Persistence of viral RNA in the central nervous system of mice inoculated with MHV-4, p. 327–332. *In* H. Laude and F. J. Vautherot (ed.), Coronaviruses. Plenum Press, Inc., New York, N.Y.
10. **Fleming, J. O., F. I. Wang, M. D. Trousdale, D. R. Hinton, and S. A. Stohlman.** 1993. Interaction of immune and central nervous systems: contribution of antiviral Thy-1^+ cells to demyelination induced by coronavirus JHM. Reg. Immunol. **5:**37–43.
11. **Frankowski, H., M., Missortten, F.-A. Fernandez, I. Martinou, P. Michel, R. Sadoul, and J. C. Martinou.** 1995. Function and expression of the Bcl-x gene in the developing and adult nervoiu system. NeuroReport **6:**1919–1921.
12. **Gonzalez-Garcia, M., I. Garcia, L. Ding, S. O'Shea, L. H. Boise, C. B. Thompson, and G. Nunez.** 1995. Bcl-x is expressed in embryonic and postnatal neural tissues and functions to prevent neuronal cell death. Proc. Natl. Acad. Sci. USA **92:**4304–4308.
13. **Gonzalez-Garcia, M., R. Perez-Ballestero, L. Ding, L. Duan, L. H. Boise, C. B. Thompson, and G. Nunez.** 1994. Bcl-XL is the major bcl-x mRNA form expressed during murine development and its product localizes to mitochondria. Development **120:**3033–3042.
14. **Hingley, S. T., J. L. Gombold, E. Lavi, and S. R. Weiss.** 1994. MHV-A59 fusion mutants are attenuated and display altered hepatotropism. Virology **200:**1–10.
15. **Hirano, N., K. Fujiwara, S. Hino, and M. Matumoto.** 1974. Replication and plaque formation of mouse hepatitis virus (MHV-2) in mouse cell line DBT culture. Arch. Gesamte. Virusforsch. **44:**298–302.
16. **Hirano, N., N. Goto, S. Makino, and K. Fujiwara.** 1981. Persistent infection with mouse hepatitis virus JHM strain in DBT cell culture, p. 301–308. *In* V. ter Meulen, S. Siddell, and H. Wege (ed.), Biochemistry and biology of coronaviruses. Plenum Press, Inc., New York, N.Y.
17. **Hofmann, M. A., S. D. Senanayake, and D. A. Brian.** 1990. A translation-attenuating intraleader open reading frame is selected on coronavirus mRNAs during persistent infection. Proc. Natl. Acad. Sci. USA **90:**11733–11737.
18. **Houtman, J. J., and J. O. Fleming.** 1996. Dissociation of demyelination and viral clearance in congenitally immunodeficient mice infected with murine coronavirus JHM. J. Neurovirol. **2:**101–110.
19. **Kalicharran, K., D. Mohandas, G. Wilson, and S. Dales.** 1996. Regulation of the initiation of coronavirus JHM infection in primary oligodendrocytes and L-2 fibroblasts. Virology **225:**33–43.
20. **Knobler, R. L., M. Dubois-Dalcq, M. V. Haspel, A. P. Claysmith, P. W. Lampert, and M. B. Oldstone.** 1981. Selective localization of wild type and mutant mouse hepatitis virus (JHM strain) antigens in CNS tissue by fluorescence, light and electron microscopy. J. Neuroimmunol. **1:**81–92.
21. **Knobler, R. L., P. W. Lampert, and M. B. A. Oldstone.** 1982. Virus persistence and recurring demyelination produced by a temperature-sensitive mutant of MHV-4. Nature **298:**279–280.
22. **Kooi, C., M. Cervin, and R. Anderson.** 1991. Differentiation of acid-pH-dependent and -nondependent entry pathways for mouse hepatitis virus. Virology **180:**108–119.
23. **Krajewski, S., M. Krajewska, A. Shabaik, H. G. Wang, S. Irie, L. Fong, and J. C. Reed.** 1994. Immunohistochemical analysis of in vivo patterns of Bcl-X expression. Cancer Res. **54:**5501–5507.
24. **Lai, M. M. C., and D. Cavanagh.** 1997. The molecular biology of coronaviruses. Adv. Virus Res. **48:**1–100.
25. **Lampert, P. W., J. K. Sims, and A. J. Kniazeff.** 1973. Mechanism of demyelination in JHM virus encephalomyelitis: electron microscopic studies. Acta Neuropathol. **24:**76–85.
26. **Lavi, E., D. H. Gilden, M. K. Highkin, and S. R. Weiss.** 1984. Persistence of mouse hepatitis virus A59 RNA in a slow virus demyelinating infection in mice as detected by in situ hybridization. J. Virol. **51:**563–566.
27. **Lavi, E., A. Suzumura, M. Hirayama, M. K. Highkin, D. M. Dambach, D. H. Silberberg, and S. R. Weiss.** 1987. Coronavirus mouse hepatitis virus (MHV)-A59 causes a persistent, productive infection in primary glial cell cultures. Microb. Pathog. **3:**79–86.
28. **Levine, B., Q. Huang, J. T. Isaacs, J. C. Reed, D. E. Griffin, and J. M. Hardwick.** 1993. Conversion of lytic to persistent alphavirus infection by the bcl-2 cellular oncogene. Nature **361:**739–742.
29. **Lin, M. T., D. R. Hinton, N. W. Marten, C. C. Bergmann, and S. A. Stohlman.** 1999. Antibody prevents virus reactivation within the central nervous system. J. Immunol. **162:**7358–7368.
30. **Liu, Y., Y. Cai, and X. M. Zhang.** 2003. Induction of caspase-dependent apoptosis in cultured rat oligodendrocytes by murine coronavirus is mediated during cell entry and does not require virus replication. J. Virol. **77:** 11952–11963.
31. **Louis, J. C., E. Magal, D. Muir, M. Manthorpe, and S. Varon.** 1992. CG-4, a new bipotential glial cell line from rat brain, is capable of differentiating in vitro into either mature oligodendrocytes or type 2 astrocytes. J. Neurosci. Res. **31:**193–204.
32. **Matthews, A. E., E. Lavi, S. R. Weiss, and Y. Paterson.** 2002. Neither B cells nor T cells are required for CNS demyelination in mice persistently infected with MHV-A59. J. Neurovirol. **8:**257–264.
33. **Matthews, A. E., S. R. Weiss, and Y. Paterson.** 2002. Murine hepatitis virus–a model for virus-induced CNS demyelination. J. Neurovirol. **8:**76–85.
34. **McNulty, S., M. Crouch, D. Smart, and M. Rumsby.** 2001. Differentiation of bipolar CG-4 line oligodendrocytes is associated with regulation of CREB, MAP kinase and PKC signaling pathways. Neurosci. Res. **41:**217–226.

35. **Merry, D. E., and S. J. Korsmeyer.** 1997. Bcl-2 gene family in the nervous system. Annu. Rev. Neurosci. **20:**245–267.
36. **Motoyama, N., F. Wang, K. A. Roth, H. Sawa, K. Nakayama, K. Nakayama, I. Negishi, S. Senju, Q. Zhang, S. Fujii, et al.** 1995. Massive cell death of immature hematopoietic cells and neurons in Bcl-x-deficient mice. Science **267:**1506–1510.
37. **Murray, R. S., B. Brown, D. Brian, and G. F. Gabirac.** 1992. Detection of coronavirus RNA and antigen in multiple sclerosis brain. Ann. Neurol. **31:**525–533.
38. **Nash, T. C., and M. J. Buchmeier.** 1997. Entry of mouse hepatitis virus into cells by endosomal and nonendosomal pathways. Virology **233:**1–8.
39. **Pasick, J. M., and S. Dales.** 1991. Infection by coronavirus JHM of rat neurons and oligodendrocyte type 2 astrocyte lineage cells during distinct developmental stages. J. Virol. **65:**5013–5028.
40. **Perlman, S., G. Jacobsen, L. A. Olson, and A. Afifi.** 1990. Identification of the spinal cord as a major site of persistence during chronic infection with a murine coronavirus. Virology **175:**418–426.
41. **Perlman, S., and D. Reis.** 1987. The astrocyte is a target cell in mice persistently infected with mouse hepatitis virus, strain JHM. Microbiol. Pathog. **3:**309–314.
42. **Ramakrishna, C., C. C. Bergmann, R. Atkinson, and S. A. Stohlman.** 2003. Control of central nervous system viral persistence by neutralizing antibody. J. Virol. **77:**4670–4678.
43. **Rowe, C. L., S. C. Baker, M. J. Nathan, and J. O. Fleming.** 1997. Evolution of mouse hepatitis virus: detection and characterization of spike deletion variants during persistent infection. J. Virol. **71:**2959–2969.
44. **Sawicki, S. G., J. H. Lu, and K. V. Holmes.** 1995. Persistent infection of cultured cells with mouse hepatitis virus (MHV) results from the epigenetic expression of the MHV receptor. J. Virol. **69:**5535–5543.
45. **Schwartz, T., L. Fu, and E. Lavi.** 2001. Programmed cell death in MHV-induced demyelination. Adv. Exp. Med. Biol. **494:**163–167.
46. **Schwartz, T., L. Fu, and E. Lavi.** 2002. Differential induction of apoptosis in demyelinating and nondemyelinating infection by mouse hepatitis virus. J. Neurovirol. **8:**392–399.
47. **Stewart, J. N., S. Mounir, and P. J. Talbot.** 1992. human coronavirus gene expression in the brains of multiple sclerosis patients. Virology **191:**502–505.
48. **Stohlman, S. A., C. C. Bergmann, and S. Perlman.** 1999. Mouse hepatitis virus, p. 537–557. *In* R. Ahmed and I. Chen (ed.), Persistent viral infections. Wiley, New York, N.Y.
49. **Stohlman, S. A., A. Y. Sakaguchi, and L. P. Weiner.** 1979. Characterization of the cold-sensitive hepatitis virus mutants rescued from latently infected cells by cell fusion. Virology **98:**448–455.
50. **Stohlman, S. A., A. Y. Sakaguchi, and L. P. Weiner.** 1979. Rescue of a positive-stranded RNA virus from antigen-negative neuroblastoma cells. Life Sci. **24:**1029–1036.
51. **Stohlman, S. A., and L. P. Weiner.** 1978. Stability of neurotropic mouse hepatitis virus (JHM) strain during chronic infection of neuroblastoma cells. Arch. Virol. **57:**53–83.
52. **Van Dinter, S., and W. F. Flintoff.** 1987. Rat glial C6 cells are defective in murine coronavirus internalization. J. Gen. Virol. **68:**1677–1685.
53. **Wang, F. I., S. A. Stohlman, and J. O. Fleming.** 1990. Demyelination induced by murine hepatitis virus JHM strain (MHV-4) is immunologically mediated. J. Neuroimmunol. **30:**31–41.
54. **Williams, R. K., G. Jiang, and K. V. Holmes.** 1991. Receptor for mouse hepatitis virus is a member of the carcinoembryonic antigen family of glycoproteins. Proc. Natl. Acad. Sci. USA **88:**5533–5536.
55. **Wu, G. F., and S. Perlman.** 1999. Macrophage infiltration, but not apoptosis, is correlated with immune-mediated demyelination following murine infection with a neurotropic coronavirus. J. Virol. **73:**8771–8780.
56. **Wu, G. F., A. A. Dandekar, L. Pewe, and S. Perlman.** 2000. CD4 and CD8 T cells have redundant but not identical roles in virus-induced demyelination. J. Immunol. **165:**2278–2286.
57. **Yokomori, K., and M. M. Lai.** 1992. Mouse hepatitis virus utilizes two carcinoembryonic antigens as alternative receptors. J. Virol. **66:**6194–6199.
58. **Zhang, X. M., K. G. Kousoulas, and J. Storz.** 1991. Comparison of the nucleotide and deduced amino acid sequences of the S genes specified by virulent and avirulent strains of bovine coronaviruses. Virology **183:**397–404.