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Coronavirus mouse hepatitis virus (MHV)-A59 causes a persistent, productive infection in primary glial cell cultures

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MHV-A59 causes a chronic demyelinating disease in mice which is accompanied by persistence of viral genome in white matter. As part of the investigation into the mechanism of viral persistence, infection of glial cells, probable targets for chronic infection, was studied by the use of mixed glial, enriched oligodendrocyte and enriched astrocyte cultures. Following MHV-A59 infection *in vitro*, approximately 10% of oligodendrocytes and 30% of astrocytes expressed viral antigens in the absence of overt cytopathic effect. All cultures released infectious virus for the lifetime of the cultures, for at least 45 days in the case of mixed glial cultures. Cultures derived from previously infected mice were similar to those infected *in vitro* with respect to percentage of cells expressing viral antigen and levels of infectious virus produced. These results show (1) that glial cells are early sites of infection *in vivo* as well as sites of infection in *in vitro* cultures, and (2) that glial cells support a non-lytic but productive infection *in vitro* and thus may contribute to viral persistence *in vivo*.

Key words: Coronavirus; mouse hepatitis virus (MHV-A59); oligodendrocytes; astrocytes; persistent infection.

Introduction

Mouse hepatitis virus strain A59 (MHV-A59), a neurotropic murine coronavirus, is an enveloped positive stranded RNA virus.¹ After intranasal or intracerebral inoculation of mice, MHV-A59 produces mild acute encephalitis with infection of selected regions of the central nervous system (CNS) and subsequent chronic demyelination.²⁻⁴ MHV-A59 infection of mice has been used as an experimental model system to study virus-induced demyelination; this may be important in the understanding of the pathogenesis of human demyelinating diseases such as multiple sclerosis (MS) and acute disseminated encephalomyelitis (ADEM).

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Studies with MHV-JHM, another neurotropic strain of MHV, have suggested that MHV-induced demyelination is the result of lytic infection of oligodendrocytes, the myelin producing cells.^{5,6} We have previously shown that although infectious MHV-A59 and viral antigens are extremely difficult, if not impossible, to detect after the first few weeks post-infection, viral genome persists in the white matter of infected mice during the chronic demyelinating stages of the disease.⁷ These findings, along with the observation that MHV-A59 grows preferentially in non-neuronal (as opposed to neuronal) cells in dissociated spinal cord cultures,⁸ suggest that glial cells may be the site of MHV-A59 persistence during chronic demyelination. A recent report showed that MHV-A59 can productively infect astrocytes *in vitro*.⁹

Thus, as part of our approach to understanding the mechanism of MHV-A59 persistence and its possible relationship to demyelination, we studied the interaction between MHV-A59 and oligodendrocytes and astrocytes *in vitro* using enriched cultures of each cell type, derived from mice. We have used these cultures to complement ongoing *in vivo* studies for several reasons: (1) the interaction between virus and cell can be studied isolated from other host factors such as the immune system; (2) unambiguous identification of cell type with the cell-specific markers is more easily accomplished *in vitro* than *in vivo*; and (3) a high percentage of a specific cell type as well as a large number of cells are obtained in each culture; thus oligodendrocytes and astrocytes can be studied separately. We report here that astrocytes and oligodendrocytes are early sites of MHV-A59 infection *in vivo* and that glial cells support persistent productive MHV-A59 infection *in vitro*.

Results

Expression of viral antigen in glial cell cultures.

Mixed glial, enriched oligodendrocyte and enriched astrocyte cultures were derived either from newborn or 3 week old C57BL/6 mice as described in 'Materials and methods'.¹⁰⁻¹² Such cultures were infected with MHV-A59 at multiplicity of infection (MOI) of 1 tissue culture infectious dose (TCID)₅₀/cell and 3 days post-infection were stained by double immunofluorescence for viral antigen and cell-specific markers. Approximately 30% of glial fibrillary acidic protein (GFAP)-positive cells (astrocytes)¹³ and 10% of galactocerebroside (GalC)¹⁴ positive cells (oligodendrocytes) expressed cytoplasmic viral antigens. Figure 1 shows an example of cells that were positive for GalC and viral antigens. Viral antigen was not detected in control mock-infected cultures or in cultures that were incubated with normal mouse serum. There was no obvious cytopathic effect (CPE) in infected cultures observed with the light microscope (during a period of 45 days) as compared to uninfected cultures and there was no evidence of syncytia formation which is observed in lytic infections of MHV-A59 *in vitro* in 17Cl-1 mouse fibroblasts.¹⁵ Thus at least some oligodendrocytes and some astrocytes can be infected by MHV-A59 *in vitro*.

Because only 10% of the oligodendrocytes and 30% astrocytes contained viral antigen we wanted to determine whether all cells could be infected. Thus mixed glial cultures were infected with MHV-A59 at MOIs of 0.1, 1, 5, 50 TCID₅₀/cell. The percentage of infected cells did not significantly increase with the higher multiplicities. There was no obvious CPE during an observation period of 1 week.

To determine whether glial cells may be infected by MHV-A59 *in vivo* as well, as *in vitro*, 5-day old mice were infected by intracerebral inoculation with 400 TCID₅₀ of MHV-A59 (a lethal dose for suckling mice) and sacrificed one day later. Mixed glial

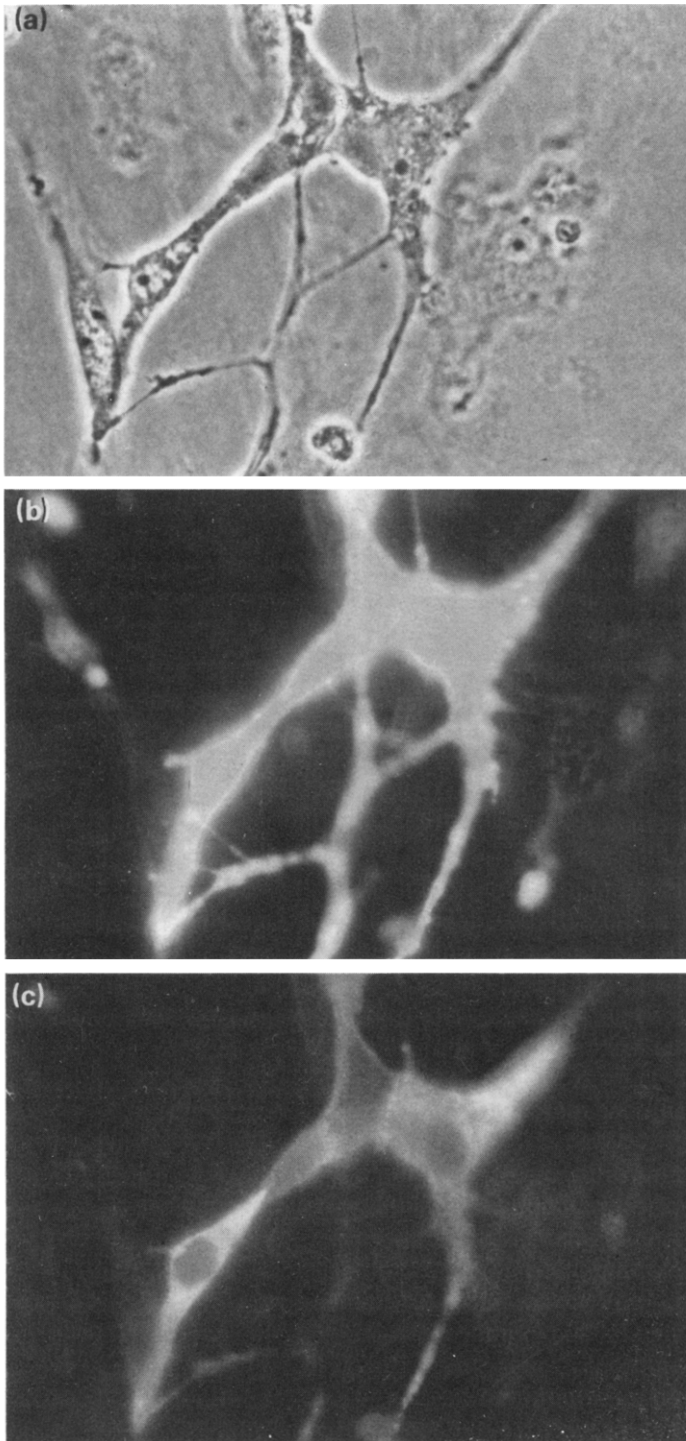


Fig. 1. Expression of MHV-A59 antigen in infected oligodendrocytes. Oligodendrocyte enriched cultures were derived from 3 week old C57BL/6 mice by Percoll density gradient centrifugation⁵ and at 1 day after isolation cultures were infected with MHV-A59 (MOI=1 TCID₅₀/cell). At 3 days post-infection cells were washed and incubated with rabbit polyclonal anti-galactocerebroside (GalC) (1:100 dilution) and rhodamine-conjugated secondary antibody, then fixed with acid alcohol and incubated with mouse polyclonal anti-MHV-A59 (1:10 dilution) and fluorescein-conjugated secondary antibody. When viewed with phase contrast (a), rhodamine (b), and fluorescein (c), optics, positive cytoplasmic staining of MHV-A59 antigen (c) was seen in cells that appear morphologically as oligodendrocytes (a) and stained with anti-GalC (b). Cells observed in (a) that are not stained in (b) and (c) are uninfected and not oligodendrocytes. They are probably contaminating fibroblasts and astrocytes.

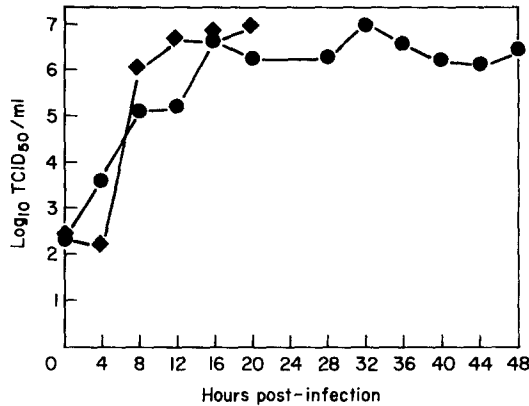


Fig. 2. Growth kinetics of MHV-A59 in glial cells as compared to 17Cl-1 cells. T75 flasks containing either 17Cl-1 cells or mixed glial cells (two weeks after extraction from newborn C57BL/6 mice) were infected with MHV-A59 at MOI=1 TCID₅₀/cell. Samples of supernatant were removed at the indicated intervals and assayed for infectious virus in a TCID₅₀ assay in mouse fibroblasts. All time points were assayed in duplicate cultures; the values shown above are averages. (◆), 17Cl-1 cells. (●), glial cells

cultures were derived and one day later were stained by double immunofluorescence with viral antiserum and cell specific markers as described above. Again both cell types were infected by the virus; viral antigens were detected in approximately the same percentage of cells as in cultures that were infected *in vitro*. Thus astrocyte and oligodendrocytes are early sites for MHV-A59 infection *in vivo*.

Production of infectious virus by glial cell cultures

Since both oligodendrocytes and astrocytes contained viral antigen in the absence of CPE we wanted to determine if infectious virus was produced by these cells. Thus, titers of infectious virus in the supernatants of infected cultures were determined using a tissue culture infectious dose (TCID)₅₀ assay. These results are shown in Figs 2 and 3. The kinetics of infectious virus production were measured in the first 48 hours post-infection in mixed glial cultures and compared with kinetics in 17Cl-1 mouse fibroblasts, the cell line used for growth of the virus. In the 17Cl-1 cells infected at MOI=1 TCID₅₀/cell, a productive lytic infection destroyed the cells by 20 hours post-infection when virus production was maximal (Fig. 2). In glial cell cultures, at both MOI=1 TCID₅₀/cell (Fig. 2) and MOI=50 TCID₅₀/cell (data not shown), the kinetics of production of infectious virus were similar to the kinetics in 17Cl-1 cells. However the glial cultures were strikingly different in that there was no CPE. The fact that similar titers of virus were produced by cultures infected at MOI of 1 or 50 TCID₅₀/cell is consistent with the fact that the percentage of cells expressing viral antigen also did not increase at higher MOIs (see above).

Since the glial cells were not destroyed by infection, we continued to culture these cells. As shown in Fig. 3, in the *in vitro* infected mixed glial cultures, there was a continual but variable release of virus at moderate levels for at least 45 days post-infection. Similarly moderate levels of infectious virus were measured in supernatants from oligodendrocyte cultures during a 17-day period, and in the supernatants from astrocyte cultures during a 14-day period. As shown in Fig. 4, the continual virus release was also observed in mixed glial cell cultures that had been derived from pre-infected mice 1 day after infection. Thus, *in vitro* glial cultures either infected *in vitro* or derived from pre-infected mice produced infectious virus as long as the cultures

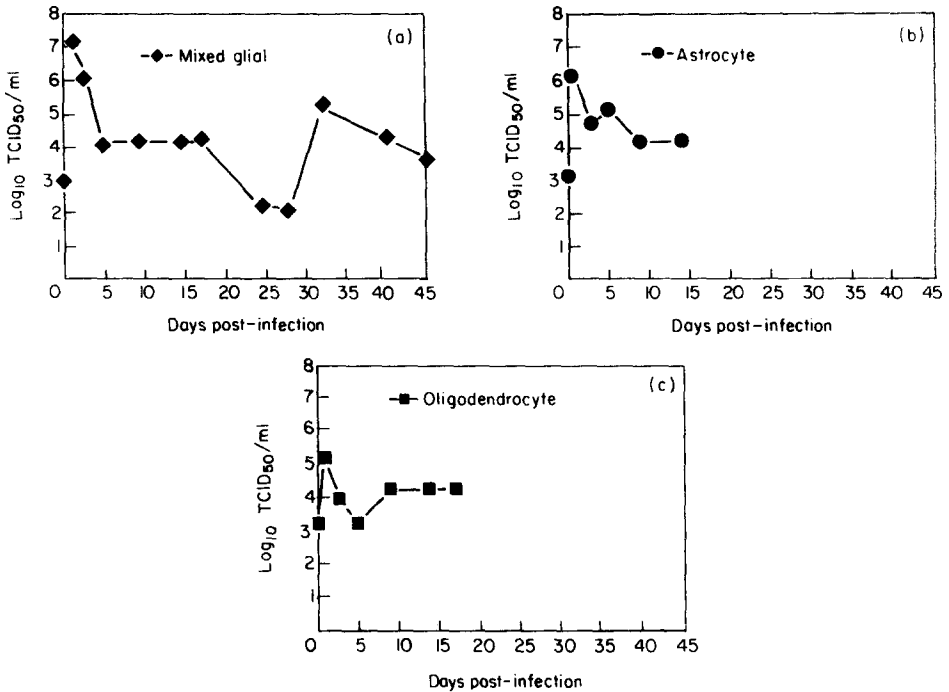


Fig. 3. Titers of MHV-A59 in glial cell cultures infected *in vitro*. Mixed glial cell cultures as well as oligodendrocyte and astrocyte enriched cultures derived from newborn C57BL/6 mice were infected with MHV-A59 ($\text{MOI}=1 \text{TCID}_{50}/\text{cell}$). At the indicated intervals post infection supernatants were removed and tested for infectious virus by a TCID_{50} assay on 17Cl-1 mouse fibroblasts. (a) Mixed glial cell cultures. (b) Enriched astrocyte cultures. (c) Enriched oligodendrocyte cultures. Mixed glial cell cultures and astrocytes cultures contained approximately 5-fold more cells per ml supernatant than oligodendrocyte cultures. Thus, the amount of virus produced per cell for oligodendrocyte cultures was similar to that for the astrocyte cultures.

were maintained. There was no CPE observed in any of the cultures for the time courses monitored in Figs 3 and 4, that is up to 45 days for the mixed glial cultures. There were no morphological changes in the cultures that could be correlated with fluctuations in viral titers released.

Discussion and conclusions

Oligodendrocytes and astrocytes are not only susceptible to MHV-A59 infection *in vitro* but are also early sites of virus infection *in vivo*. Mixed glial cultures, enriched astrocyte cultures and enriched oligodendrocyte cultures derived from newborn mice all support persistent, productive infection with MHV-A59, which occurs in the absence of CPE. (We were unable to examine viral antigens and virus production in glial cells extracted from weanling mice, after *in vivo* infection, because of technical difficulties in deriving such cultures.) Thus, the interaction of MHV-A59 with glial cells is different from that of the virus with 17Cl-1 mouse fibroblasts in that the latter cells undergo syncytia formation and subsequent cell lysis within the first day post infection.

Our results suggest that astrocytes are sites of viral persistence in the cultures. The astrocytes cultured from mouse brain divide in culture and the cultures remain > 90% pure for the duration of the experiment as assayed by staining with GFAP.^{10,11} It

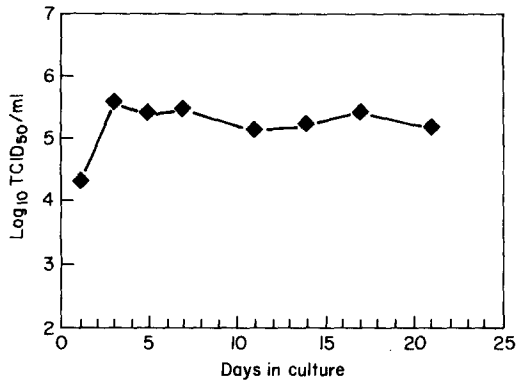


Fig. 4. Titer of MHV-A59 in glial cell cultures derived from mice that were infected *in vivo*. Mixed glial cultures were derived from 5 day old mice 1 day after intracerebral infection with MHV-A59 400 TCID₅₀/inoculum. At the times indicated, supernatants were removed and titered for infectious virus by TCID₅₀ assay on L-2 mouse fibroblasts.

is more difficult to be sure of the role of oligodendrocytes in viral persistence. Oligodendrocytes do not divide in culture; thus enriched oligodendrocyte cultures (70–80% pure at 3 days after plating) are only 20–40% GalC positive at 2 weeks after plating. Although virus production continued for the 2 weeks that the enriched oligodendrocyte cultures were maintained *in vitro* (Fig. 3) it is difficult to be sure that infectious virus was continually being produced by the oligodendrocytes as the cultures became contaminated with virus-producing fibroblasts and astrocytes. However some of the remaining oligodendrocytes (GalC positive cells) did remain positive for viral antigens.

The non-lytic nature of the interaction between MHV-A59 and glial cells may be either due to host cell factors that modify viral gene expression or to selection of variant virus defective in expression of one or more viral genes. The latter has been shown to be the case in persistent paramyxovirus infections.^{16,17} The selection of a viral mutant is less likely in MHV-A59 infected glial cells as the virus produced by these cells is lytic in 17Cl-1 cells. It is more likely that an interaction of glial host cell factors with virus results in a non-lytic infection. We are investigating viral RNA and protein expression, during MHV persistence in glial cells.

The outcome of infection of MHV in primary glial cells is dependent on the virus strain, and the age and strain of the mice from which the cultures are derived. In another study of MHV-A59 in primary glial cell cultures, infection was productive and lytic and virtually all the cells contained viral antigen.⁹ This study differed from ours in that the MHV-A59 strain used was at least 30-fold more lethal in mice and a different strain and age of mice was used to derive the cultures. In studies with JHM, a more neuropathogenic MHV strain, chronic productive infections were established in primary glial cells or enriched oligodendrocyte cultures similar to that which we described.^{18,19} It was shown that resistance to JHM infection in *in vitro* rat oligodendrocyte cultures was related to the developmental state of the cells. Perhaps the fact that even at high MOIs in our studies only 10–30% of glial cells expressed viral antigen is related to the physiological or developmental state of the cells.

The facts that viral nucleic acids are restricted to white matter during chronic MHV-A59 infection in mice⁷ and that glial cells undergo persistent productive infection *in vitro* support the hypothesis that virus persists in glial cells during chronic demyelination. Persistent infection of oligodendrocytes may cause a malfunction of the myelin

synthesis or may alter myelination by a more indirect, perhaps immune mediated mechanism. Persistent MHV infection of primary glial cells is accompanied by enhancement of H-2 class 1 antigen expression.²⁰ This could potentially lead to the recognition of infected oligodendrocytes by cytotoxic T lymphocytes that could lyse the oligodendrocytes to cause demyelination. We are currently investigating this idea.

Materials and methods

Glial cell cultures. All cultures were derived from C57BL/6 mice obtained from Jackson Laboratories (designated 'MHV-free'). Mixed glial cell cultures were derived from 3 week old mice by mincing and trypsinization of brains followed by filtration through nylon mesh and plating onto tissue culture flasks. Oligodendrocytes were purified from these brains by Percoll gradient centrifugation.¹² Alternatively, mixed glial cultures were derived from newborn mice and after 2 weeks in culture were subcultured into enriched astrocytes and enriched oligodendrocyte cultures by the procedure of McCarthy and DeVellis¹⁰ designed for rat glial cells as modified for use with mouse cells.¹¹ Briefly, this involves shaking off of the less adherent oligodendrocytes and replating of these cells onto poly-L-lysine coated cover slips (at a concentration of approximately 5×10^4 cells per cm^2) while the astrocytes remain adherent to the tissue culture plastic. In both cases infections were performed on cells at 3–4 weeks of development. In both cases, cultures were similar in terms of percentage of cells containing viral antigen and amount of infectious virus produced. Because the cells were difficult to obtain routinely from the older mice, most of the experiments were performed on cultures derived from the newborn mice. All cells were grown in Dulbecco's medium with 10% fetal calf serum at 37°C in the presence of 5% CO_2 . The purity of the cultures was determined by indirect immunofluorescence. Cells were stained either with antiserum directed against galactocerebroside (GalC), an oligodendrocyte specific marker,¹⁴ or antiserum against glial fibrillary acidic protein (GFAP), an astrocyte specific marker,¹³ followed by a secondary antibody conjugated with fluorescein or rhodamine. As defined by staining with these antisera 3 days after plating, oligodendrocyte enriched cultures consisted of more than 80% oligodendrocytes whereas astrocyte enriched cultures consisted of more than 90% astrocytes.¹¹

Infection of cultures with MHV-A59. Plaque purified MHV-A59 grown in 17Cl-1 cells was used in all infections.³ 17Cl-1 cells were infected at $\text{MOI}=1$ $\text{TCID}_{50}/\text{cell}$ and glial cultures were infected 1–3 days after plating with MOIs as indicated. At the time of infection 17Cl-1, mixed glial and astrocyte cultures contained approximately 1.5×10^5 cells per cm^2 . Enriched oligodendrocytes contained approximately 5×10^4 cells per cm^2 . After adsorption for 1 hour at room temperature cultures were washed with phosphate buffered saline three times and fed with warm media. At the indicated intervals post infection, supernatants were collected (replaced with fresh media) and tested for infectious virus by a TCID_{50} assay in 17Cl-1 or L-2 mouse fibroblasts (as indicated) as previously described.²¹ Titers were approximately the same in both cell types.

Infection in mice. Five day old C57BL/6 mice were infected intracerebrally with 400 TCID_{50} of MHV-A59. One day later brains were removed and mixed glial cultures derived by the techniques of McCarthy and DeVellis¹⁶ as described above.

Antigen detection by immunofluorescence. Cultures were stained by immunofluorescence. When staining for oligodendrocytes expressing viral antigens, cells were washed and incubated with rabbit polyclonal anti-galactocerebroside serum (1:100 dilution) and rhodamine-conjugated secondary antibody, then fixed with acetic acid/ethanol and incubated with mouse polyclonal anti-MHV-A59 (1:100 dilution) and fluorescein-conjugated secondary antibody.²⁰ When staining for astrocytes expressing viral antigens cells were fixed, stained with rabbit polyclonal anti glial fibrillary acidic protein serum (1:150) and rhodamine-conjugated secondary antibody and then stained for viral antigens as above. Cells were then viewed with phase contrast, rhodamine, and ultraviolet fluorescence optics.

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