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Short Communication

The astrocyte is a target cell in mice persistently infected with mouse hepatitis virus, strain JHM

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Mouse hepatitis virus, strain JHM (MHV–JHM), causes a late onset, clinically apparent, demyelinating encephalomyelitis in 40% of suckling C57BL/6 mice born to immunized dams. Suckling mice born to unimmunized dams rapidly succumb to an acute encephalomyelitis. MHV–JHM can be isolated from the brains and spinal cords of maternal antibody-protected mice when the late onset disease becomes clinically apparent, showing that the virus must be present in these mice when they are still asymptomatic. To determine which cells of the central nervous system (CNS) were potential reservoirs for the virus during the asymptomatic period, tissue sections were assayed simultaneously by immunoperoxidase and immunofluorescence staining for the presence of viral antigen and for glial fibrillary acidic protein (GFAP), a marker for astrocytes.[2] The results indicate that 20% (range 0–52%) of the MHV-JHM infected cells in asymptomatic mice were astrocytes. In mice symptomatic with late onset hindlimb paralysis, a higher percentage of infected cells were astrocytes. These results indicate that astrocytes are a target cell in both symptomatic and asymptomatic mice persistently infected with MHV–JHM, and suggest that the astrocyte is a potential cellular reservoir for MHV–JHM in asymptomatic mice.

Key words: Coronavirus; astrocyte; glial fibrillary protein; mouse hepatitis virus.

Introduction

Several strains of mouse hepatitis virus (MHV), a coronavirus, have the capacity to undergo latency or low level persistence in mice or rats.[3–9] We have recently described a model for viral latency and reactivation in which the JHM strain of MHV (MHV–JHM) remained dormant in mice for several weeks before causing clinical disease. Specifically, we showed that suckling C57BL/6 mice born to dams immunized with MHV–JHM were protected against the acute, invariably fatal, encephalomyelitis.[1] However, 40% of the mice developed a symptomatic, demyelinating encephalomyelitis with extensive destruction in the white matter of the brainstem and spinal cord at 35 days post infection (p. i.) (range 23–60 days p.i.). Viral antigen could be detected in most mice, whether symptomatic or not, although virus could only be isolated from mice with clinical disease.

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Table 1 Fraction of MHV infected cells which are astrocytes

Clinical disease	Days p.i.	No. mice examined	No. MHV–JHM positive cells counted	Percent cells positive for MHV–JHM and GFAP[a]
Acute encephalomyelitis (Born to unimmunized dams)	5	1	185	35
Asymptomatic mice (Born to immunized dams)	30–97	9	772	20 (00–52)
Hindlimb paralysis (Born to immunized dams)	28–60	4	663	39 (26–54)

[a] Indicates (No. positive for GFAP and MHV–JHM)/(No. positive for MHV–JHM). Range for individual mice is shown in parentheses.

Since virus persists in most mice, we have used this model to study which types of cells are infected in asymptomatic and symptomatic animals. Since viral antigen can be detected in the CNS of most mice, it is likely that the brain or the spinal cord is the source for reactivated virus. In this study, we have analyzed MHV–JHM infected cells in tissue sections from the brain and spinal cord for glial fibrillary acidic protein (GFAP), a specific marker for astrocytes[2] and compared the results to a similar analysis of cells in mice with acute encephalomyelitis or with hindlimb paralysis. The results are summarized in Table 1 and examples of infected cells positive (Fig. 1 (A)–(D)) or negative (Fig. 1 (E) and (F)) for GFAP are shown.

Acute encephalomyelitis in suckling mice born to unimmunized dams

Viral antigen was readily detected in these mice and 35% of the MHV–JHM infected cells in the mouse which was studied were also positive for GFAP (Table 1). Some of these cells had the typical morphology of astrocytes with multiple thin processes radiating out from the cell body whereas others resembled large reactive astrocytes (large cells with hypertrophied cytoplasm) (Fig. 1 (A) and (B)). The majority of infected astrocytes, however, were small cells without typical morphology and stained less darkly for GFAP. These cells presumably were astrocytes in the final stages of degeneration.

Symptomatic mice born to immunized dams

Forty percent of the mice born to immunized dams became clinically symptomatic several weeks after inoculation. Focal distributions of cells containing viral antigen were readily detected in these mice, with a large inflammatory response apparent. Approximately 39% of the MHV–JHM infected cells were also positive for GFAP (Table 1), with most of the infected astrocytes present in the white matter of the brain and spinal cord. Many of the infected astrocytes in these mice were small cells which lightly stained for GFAP, although some with the typical morphology of astrocytes were also observed.

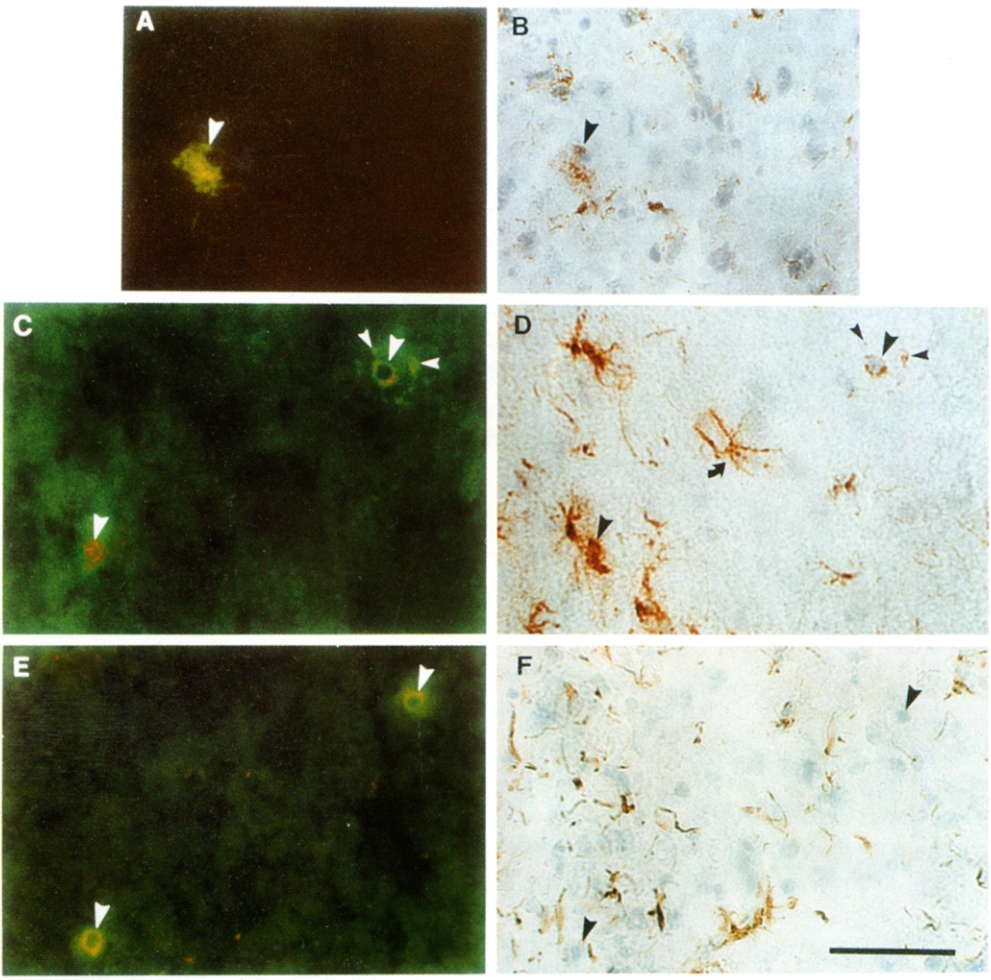

Fig. 1. (A) and (B) This frozen section, from the cerebral grey matter of a mouse with acute encephalomyelitis shows a large, reactive astrocyte (arrowheads) infected with MHV–JHM. A few isolated, uninfected astrocytic processes are also evident. The section is from a 15-day-old suckling mouse born to an unimmunized dam and inoculated at 10 days of life. This section was counterstained with hematoxylin. (C) and (D) This frozen section, from the cerebrum of an asymptomatic, maternal-antibody protected mouse inoculated 60 days prior to sacrifice, shows viral antigen present in the cytoplasm of two cells (large arrowheads) with the typical morphology of astrocytes. The processes of one of these astrocytes also contained viral antigen (small arrowheads). The majority of infected cells in all mice resembled this latter cell, although associated astrocytic processes were usually not apparent. Also shown in this figure are several uninfected astrocytes with typical morphology (one is marked with an arrow). This section was very lightly stained with hematoxylin so that the nuclei of GFAP–negative cells are not apparent. (E) and (F) This frozen section, from the cerebral white matter of a maternal antibody-protected mouse with hindlimb paralysis inoculated 30 days prior to sacrifice, shows viral antigen present in the cytoplasm of two cells which are negative for GFAP (arrows). Several astrocytes, negative for MHV–JHM, are apparent in this field. This section was counterstained with hematoxylin. (A), (C), (E)—Viral antigen identified by indirect immunofluorescence assay with polyclonal mouse anti-JHM antibody. (B), (D), (F)—Astrocytes identified by indirect immunoperoxidase assay with polyclonal rabbit anti–GFAP antibody. Bar represents 50 μm.

Asymptomatic mice born to immunized dams

A large fraction (60%) of the mice born to immunized dams and inoculated with virus developed no clinical disease. Viral antigen was present in only a few cells, and the percentage of virus positive cells also positive for GFAP varied greatly between mice, ranging from 0% to slightly over 50% (Table 1). Positive cells were usually isolated and were not surrounded by an inflammatory response, although occasional small

clusters of infected cells were observed. The infected astrocytes were present for the most part in the cerebral grey matter. As in the mice with the acute encephalomyelitis, some of the infected cells had the typical morphology of astrocytes but the majority were small cells which were less intensively stained for GFAP (Fig. 1 (C) and (D)).

No obvious clinical or histological differences were apparent between the mice with a high percentage and those with a low percentage of infected astrocytes. The great variability in the percentage may reflect an uneven spatial distribution of infected astrocytes since only a small portion of each brain was actually assayed. In some mice however, sections from different portions of the brain were analyzed and showed similar percentages of infected astrocytes, suggesting that in some cases, the different percentages reflected biological differences between infected animals. Alternatively, it is possible that a higher percentage of astrocytes was infected in all mice, but that GFAP was no longer detectable in many of the infected cells as they degenerated.

This marked variability in the percentage of infected cells which were astrocytes, coupled with our previous observation that some of the infected cells in asymptomatic mice appeared to be neurons by light microscopy,[1] is consistent with a model in which MHV–JHM has a tropism for several cells in the CNS. In this model, the particular cell type infected would not be critical for the maintenance of the persistent infection. Another explanation, based on the results of Massa *et al.*[10] in dissociated Lewis rat neuronal cultures, would be that the astrocyte is the primary target cell for MHV–JHM.

In mice with the acute demyelinating disease caused by MHV–JHM, virus or viral antigen is present in glial cells, some of which were clearly identified as oligodendrocytes or astrocytes by light or electron microscopy.[11–13] Our results show that astrocytes are also a target in persistently infected mice. These observation are consistent with published reports showing that MHV–JHM has a tropism for astrocytes in dissociated mouse spinal cord cultures,[14,15] and that MHV–JHM may persist for long periods of time in primary cultures of mouse or Lewis rat astrocytes.[10,15]

The majority of MHV–JHM infected cells in the CNS were not further identified in this study. It remains important to identify what other types of CNS cells are infected in both symptomatic and asymptomatic mice, but our attempts to do so using cell-specific markers for neurons and oligodendrocytes[16–20] have been unsuccessful thus far.

Materials and methods

Animals. Pathogen-free C57BL/6 mice (Jackson Laboratories) were immunized and their offspring inoculated with MHV–JHM as described previously.[1]

Cells and viruses. MHV–JHM, kindly provided by Dr S. Weiss, was grown in BALB/c 3T3–17CL1 cells and titered in L–2 cells as previously described.[1]

Antibody preparations. Rabbit polyclonal antibody to glial fibrillary acidic protein was the generous gift of Dr Lawrence Eng. Mouse monoclonal antibody to the nucleocapsid protein of MHV–JHM (IgG2B/k antibody) was kindly provided by Dr Julian Leibowitz. Hyperimmune mouse serum was obtained by repeated immunizations of MHV–JHM in Freund's adjuvant.

Biotinylation of antibody. The monoclonal anti-MHV–JHM antibody was biotinylated by standard procedures.[21]

Immunoperoxidase and immunofluorescence assays on same sections. In preliminary experiments, we optimized conditions of sectioning and fixation and antibody concentration. We used frozen sections, prepared as previously described,[1] in order to maximize signal detection

of viral antigen. We used thin sections (6–8 microns) to minimize the likelihood of fortuitous overlap of labels. Initial experiments were performed with methanol fixation, whereas later experiments were done with PLP fixative (2% paraformaldehyde, 0.075 M lysine, 0.01 M sodium periodate, and 0.037 M phosphate buffer, pH 7.5).[22] We also found that the presence of biotinylated antibody did not affect the subsequent immunofluorescent analysis, in agreement with previous reports.[23] In control experiments, anti-MHV–JHM antibody did not react with uninfected brains.

In the first set of experiments, sections were prepared and fixed with methanol containing 0.3% hydrogen peroxide. Slides were treated with normal horse serum, and then simultaneously with the biotinylated mouse monoclonal anti-MHV–JHM antibody (1:25) and rabbit anti-GFAP antibody (1:100) for 2 hours. The slides were treated with Vectastain ABC (Vector Laboratories) used according to the manufacturer and then developed by exposure to diaminobenzidine (DAB) (0.5 mg/ml) in hydrogen peroxide (0.01%). After washing and counterstaining with hematoxylin, the samples were treated with FITC-conjugated goat anti-rabbit antibody (Cappel Laboratories). Anti-MHV–JHM staining was not blocked by prior treatment with non-specific IgG2b/k antibody (Litton Bionetics).

In the second set of experiments, sections were fixed with PLP solution.[28] The sections were treated sequentially with 20% normal goat serum, with a mixture of hyperimmune polyclonal mouse anti-MHV–JHM (1:25) and rabbit anti-GFAP antibodies (1:100) and then with horseradish peroxidase conjugated goat anti-rabbit antibody. Development and counterstaining were as above. The sections were finally treated with FITC-conjugated goat anti-mouse antibody (1:50 or 1:100) (Cappel Laboratories). No fluorescent cells were observed if the mouse anti-MHV–JHM serum was replaced by normal mouse serum.

Slides were air dried, mounted and examined using the Olympus BH–2 microscope by both standard light and fluorescent microscopy. To count cells, each section was scanned for cells containing virus. Once a cell was determined to be positive for virus, the same cell on the section was examined under the other phase. Thus in the second set of experiments, virus-specific cells were initially detected by fluorescent microscopy, and then analyzed for the presence of GFAP by light microscopy. Two to six sections were assayed for each mouse. Some mice were assayed by both methods described above and the results were very similar. In all cases, questionable cells were counted as negative, so that the fraction of virus-specific cells which are astrocytes may in fact be higher than reported above.

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