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Mouse Hepatitis Virus Type 4 Infection of Primary Glial Cultures from Genetically Susceptible and Resistant Mice†

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Mouse hepatitis virus type 4 infection of primary glial cultures, which consisted principally of astrocytes (marked by glial fibrillary acidic protein) from encephalitis-susceptible BALB/c or F_1 (BALB/c × SJL/J) hybrid mice and resistant SJL/J mice, was studied. Primary neuron cultures from BALB/c and F_1 hybrid mice were previously shown to be permissive and were destroyed within 5 days by infection with mouse hepatitis virus type 4, whereas neurons from SJL/J mice were fully resistant. In contrast, in the present study a chronic infection was established and maintained for up to 18 days in glial cultures from all three mouse strains. Infected SJL/J mouse glial cultures produced 10- to 50-fold less infectious virus and showed less cytopathic effect than did cultures from either infected BALB/c or F_1 hybrid mice. Cytopathic effect was evident initially in cells from all three strains, and continued virus production occurred in the presence of limited additional cytopathic effect. These results were not due to the production of detectable levels of interferon. This study showed that SJL/J mouse primary glial cultures were permissive for mouse hepatitis virus type 4 infection whereas SJL/J primary neuron cultures were not, and that there was an early lytic phase of infection followed by chronic infection in all three strains.

Infection of several inbred strains of mice by intracerebral inoculation with the neurotropic murine coronavirus mouse hepatitis virus type 4 (MHV-4) (JHM strain) leads to the development of an acute necrotizing encephalomyelitis, with death occurring 3 to 7 days after infection (2, 8, 16, 17). Death is caused by infection and loss of neuronal cells. In contrast, SJL/J mice are resistant to such an intracerebral inoculation (8, 14). The resistance of SJL/J mice to MHV-4 is inherited as a single autosomal recessive trait, independent of the *H-2* complex, and F_1 hybrid (BALB/c × SJL/J or SJL/J × BALB/c) mice are fully susceptible to fatal MHV-4 disease (8). After intracerebral inoculation, brain tissue from susceptible BALB/c mice yields more infectious virus than does that from resistant SJL/J mice (8), suggesting that differences in host survival might reflect differences in virus replication. It was shown that primary cultures of BALB/c mouse neurons replicated MHV-4 efficiently, correlating with neuronal destruction in vivo and death of the host. In contrast, SJL/J mouse neurons did not replicate MHV-4, correlating with survival of the host (8). Thus, the recessive gene controlling resistance to MHV-4 was expressed at the level of the SJL/J neuronal cell (8). Virus replication detected in infected SJL/J mouse brains was probably occurring mostly in nonneuronal brain cells.

In the present study, the replication of MHV-4 in primary cultures of nonneuronal brain cells (principally astrocytes) derived from either BALB/c, SJL/J, or F_1 hybrid (BALB/c × SJL/J) mice was compared. It was shown that SJL/J glial cells, unlike SJL/J neuronal cells, are productively infected with MHV-4 but produce less virus than glial cells from BALB/c or F_1 hybrid mice. Furthermore, it was shown that the initial infection established in glial cells from these three mouse strains continues chronically, shedding infective virus in the presence of limited additional cytopathic effect (CPE) and the absence of detectable interferon.

MATERIALS AND METHODS

Virus. Wild-type MHV-4 (JHM strain), originally obtained from L. Weiner (Department of Neurology and Microbiology, University of Southern California, Los Angeles), was plaque-purified three times in NCTC 1469 cells and prepared as a virus stock containing 5×10^5 PFU/ml in L24-1 cells (6). Glial cell cultures were infected at a multiplicity of 0.5 in wells and 0.1 in flasks. Virus yields from infected glial cell cultures were determined by plaque assay on L24-1 cells as previously described (8). The level of detection was 10 PFU/ml.

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Mice. BALB/c St, SJL/J, and F_1 hybrid (BALB/c × SJL/J) mice were bred in the vivarium at the Scripps Clinic and Research Foundation and used as newborns.

Cell culture. Primary glial cell cultures of mouse brain tissue were obtained by an adaptation of the procedure used by McCarthy and de Vellis (10) for rats. Newborn mice were decapitated with a sharp razor blade. The brains from 12 to 20 mice were removed, and the cerebral hemispheres were separated from the brain stem and collected in DME-F12 medium containing 10% fetal calf serum that was not heat inactivated. The tissue was transferred into a Nitex 210 nylon mesh bag, and the cells were mechanically dissociated by gently stroking the bag with a glass rod. This cell suspension was passed by gravity sequentially through 60- and 100-mesh stainless steel strainers, which were washed with 5 ml of fetal calf serum. The cell suspension and washes were poured into a centrifuge tube and spun at 800 rpm for 5 min. Cells were counted and checked for viability by trypan blue exclusion. They were resuspended at a concentration of 10^7 viable cells per ml, and 4 ml of this suspension was plated in each 25-cm^2 flask. Alternatively, 2 ml of this suspension (10^7 cells per ml) was plated per well on glass cover slips in a six-well plate. The cultures were fed by exchanging their volume (4 or 2 ml, respectively) with fresh medium every 2 to 3 days throughout the experiments. The cultures were incubated in a 5% CO_2 atmosphere at 37°C. Cultures were infected 14 days after having been established by completely removing the medium, washing the cells with phosphate-buffered saline, and incubating them with virus for 1 h at 37°C. After infection, the cultures were washed three times with phosphate-buffered saline and refed with 4 or 2 ml of DME-F12 medium containing 10% fetal calf serum that was not heat inactivated. At various times after the final wash throughout the 18 days after infection, 2-ml portions were saved when the medium was removed and changed. They were frozen and stored at −70°C until assayed for either infectious virus yield or interferon titer.

Interferon assay. Interferon was assayed by a vesicular stomatitis virus (VSV) plaque reduction assay on L-929 cells, with each sample assayed in duplicate (13). Briefly, the assay consists of plates on which a known amount of VSV is plated to give 100 plaques. Control plates were preincubated with a known dilution of an interferon standard (G002-904-511; kindly supplied by the National Institute of Allergy and Infectious Diseases) to reduce the number of VSV plaques by 50% (1 U of interferon). Test plates were preincubated with serial twofold dilutions of unknown supernatant fluid for interferon assay. The supernatant fluid was ultracentrifuged for 1 h at 30,000 rpm to pellet infectious MHV-4 virus. The level of detection was 4 IU of interferon per ml.

Identification of cells and immunochemical labeling of MHV-4 antigens. Cultures on glass cover slips were assayed for the presence of neuronal cells by detecting plasma membrane receptors for tetanus toxin (11) with tetanus toxoid, for oligodendrocytes by detecting surface galactocerebroside (12), and for astrocytes by demonstrating cytoplasmic glial fibrillary acidic protein (GFAP) (3, 5). The cultures were either unfixed or fixed with acid-alcohol, acetone, or 4% paraformaldehyde–0.5% glutaraldehyde in cacodylate buffer (7, 8). Antibodies to tetanus toxoid were generated by immunizing rabbits in this laboratory. Antibodies to galactocerebroside were generously provided by M. Rapport (New York State Psychiatric Institute, New York), and antibodies to GFAP were generously provided by L. Eng (Veterans Administration Hospital, Palo Alto, Calif.). These reagents were characterized by absorption and blocking studies with their specific substrates (tetanus toxoid, galactocerebroside, or GFAP).

For detecting MHV-4 antigens, the cultures were fixed with 4% paraformaldehyde–0.5% glutaraldehyde in cacodylate buffer (7). They were rinsed with phosphate-buffered saline and incubated with monospecific antibodies to purified MHV-4 virions or antigens that were prepared in rabbits (kindly supplied by K. Holmes, Uniformed Services University of the Health Sciences, Bethesda, Md.) or in mice by immunization in this laboratory or kindly provided by the resource program of the National Cancer Institute, Bethesda, Md. Secondary antibodies consisted of sheep antibody to mouse immunoglobulins or goat antibody to rabbit immunoglobulins conjugated with rhodamine or fluorescein isothiocyanate; both were prepared and characterized in this laboratory.

RESULTS

Characterization of cell populations. Brain cell cultures from newborn mice yielded a confluent monolayer of cells in 11 to 14 days. A dominant morphological type was present; a large, flattened cell with irregular borders, occasionally showing stellate processes. These cells contained GFAP in their cytoplasm and processes (Fig. 1) and represented more than 98% of the cells present. Occasionally there were cells of neuronallike morphology with several promi-

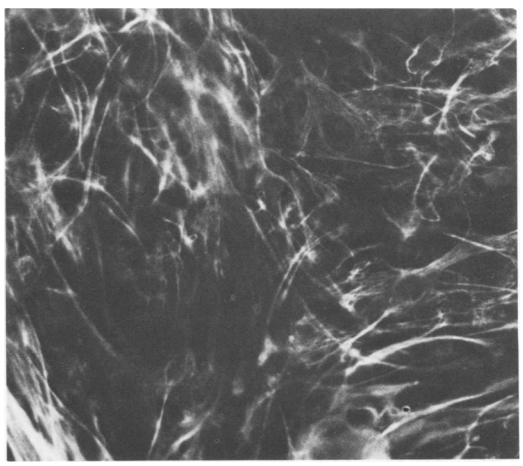

FIG. 1. GFAP labeled by immunofluorescence in processes of cultured primary glial cells derived from newborn SJL/J mouse brains, identifying these cells as astrocytes. This was the dominant cell type present in cultures derived from BALB/c, F_1 hybrid (BALB/c × SJL/J), and SJL/J mice after 2 weeks in culture (×320).

nent processes and phase-dark cells that had elaborate nets of processes resembling oligodendrocytes. However, tetanus toxoid or galactocerebroside surface-positive cells were rarely found with the appropriate reagents on unfixed or on acid-alcohol-, acetone-, or paraformaldehyde–glutaraldehyde-fixed cover slips 2 weeks after establishing the cultures. There were no differences in cell type noted in labeling cultures derived from BALB/c, SJL/J, and F_1 mice.

Replication of MHV-4 in primary glial cell cultures. Infected glial cell cultures from BALB/c, SJL/J, and F_1 hybrid mice all showed CPE, consisting of foci of fused cells forming multinucleated giant cells (syncytia), within the first 24 h after infection with MHV-4. It is of interest to note that the syncytia that formed in the SJL/J-derived cultures were small and generally limited to the size reached in the first 24-h period (Fig. 2A). In contrast, the syncytia that formed in the BALB/c- and F_1 hybrid-derived cultures were larger (Fig. 2B) than those found in the SJL/J-derived cultures and continued to expand somewhat afterward.

At 24 h after infection, SJL/J-derived cultures yielded 4×10^3 PFU of virus per ml, whereas BALB/c-derived cultures yielded 6×10^4 PFU/ml and F_1 hybrid-derived cultures yielded 3×10^5 PFU/ml (Table 1). Virus production was proportional to the extent of CPE observed in these different cultures.

Although syncytia were present in infected cultures from both strains of mice, their extension and the appearance of new syncytia was limited after the first 48 h (Fig. 3). As many as 1 to 2% of the cells in infected cultures were labeled for MHV-4 antigens between 4 and 18 days after infection. The possibility that giant cell formation in these infected cultures was limited by interferon production was investigated. Supernatant fluids at 1, 4, 7, 14, and 18 days after infection were tested for interferon by the VSV plaque reduction assay, and interferon levels were less than 4 IU/ml.

Immunofluorescent staining of cells for MHV-4 antigens and GFAP. At 72 h after infection with MHV-4 virus, when the infected cultures displayed CPE, BALB/c, F_1, and SJL/J tissue cultures were fixed in acetone and reacted sequentially with mouse antibodies to MHV-4 antigens and fluorescein-conjugated sheep anti-mouse immunoglobulin and then with rabbit

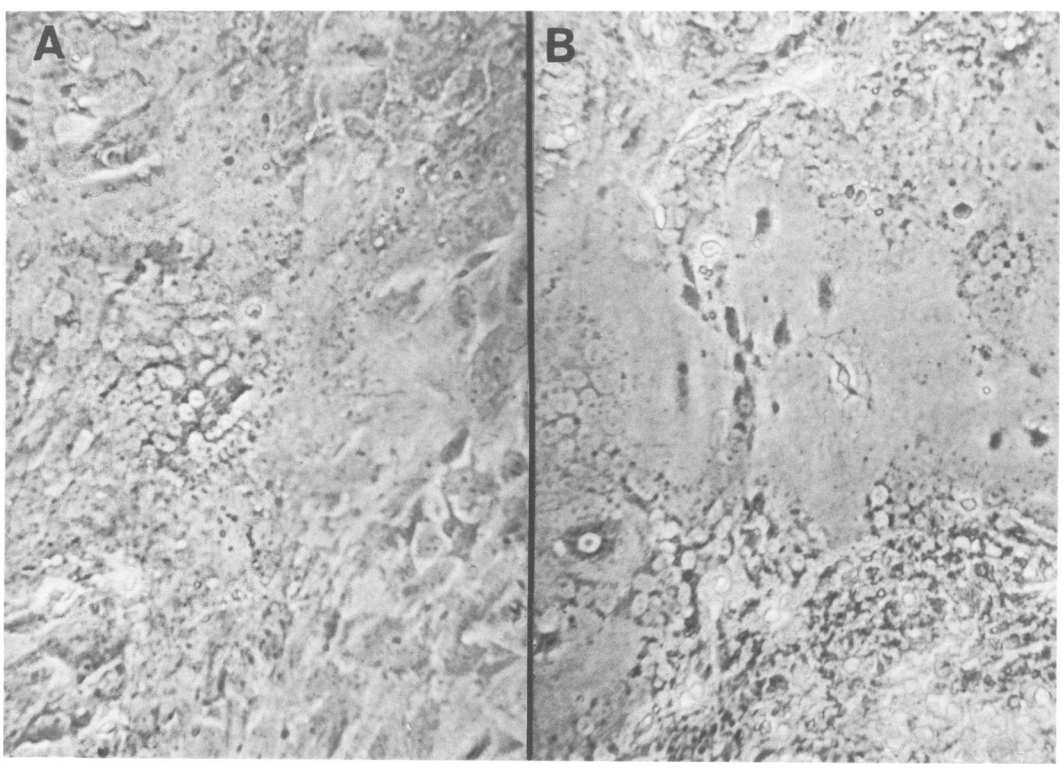

FIG. 2. Primary glial cells infected with MHV-4 at a multiplicity of 0.5 developed syncytia 24 h after infection. This CPE was less widespread in SJL/J-derived glial cells (A) than in BALB/c-derived glial cells (B) or F_1 hybrid-derived glial cells (not shown) (×400).

TABLE 1. Replication of MHV-4 in cultured mouse glial cells[a]

Time postinfection (h)	Virus titer (PFU/ml) in supernatant fluid from cells of strain:		
	BALB/c	SJL/J	F_1 (BALB/c × SJL/J)
0	0	0	0
12	5×10^3	4×10^2	6×10^4
24	6×10^4	4×10^3	3×10^5
48	1×10^4	1×10^3	2×10^5

[a] Data given are the mean values for three dishes at each time point for each strain. Each assay plate was run in duplicate. The multiplicity of infection was 0.1.

antibodies to GFAP and rhodamine-conjugated goat anti-rabbit immunoglobulin (Fig. 4). Foci of cells containing MHV-4 antigens were present in all cultures. In SJL/J cultures, single infected cells and small syncytia were present (Fig. 4A), whereas in BALB/c and F_1 cultures infected cells were usually found as syncytia involving multiple cells (Fig. 4B). In SJL/J cultures, GFAP was expressed in the same cells that contained MHV-4 antigens (Fig. 4C). In contrast, in the BALB/c and F_1 cultures, GFAP expression was decreased in some cells in which MHV-4 antigens were found (Fig. 4D). Uninfected cultures of similar age in vitro showed that GFAP-positive cells accounted for more than 98% of the cells present.

DISCUSSION

We established primary cultures of mouse glial cells from the brain tissue of newborn mice. The cultures were composed primarily of astrocytes (Fig. 1) as identified by GFAP (3, 5). We were unable to detect oligodendrocytes or neurons in these cultures.

Primary glial cultures from strains of mice genetically susceptible (BALB/c or F_1 hybrid) or resistant (SJL/J) to MHV-4-induced fatal encephalomyelitis all supported MHV-4 virus replication. The yield of virus was 10- to 50-fold less in the resistant SJL/J glial cells, and there was also less CPE (Fig. 2). Astrocytes from both resistant and susceptible animals were chronically infected throughout an 18-day period in culture (Fig. 3).

The continued release of infectious virus into the supernatant medium indicated a continuously productive virus infection of these cells. A large proportion of the cells in cultures from each mouse strain did not show CPE after the first 48 h of infection despite the continued release of infectious virus into the supernatant medium. The survival of these cells during chronic infection was not mediated by interferon, since interferon levels were below the level of detection in the supernatant medium when assayed for plaque reduction activity on VSV-infected mouse L cells. MHV-4 infection does not appear to induce detectable levels of interferon in several in vitro systems, including neuroblastoma cells (15) and macrophages (Knobler et al., manuscript in preparation). Thus, the mechanism limiting the extent of CPE in vitro despite the continued production of infectious virus is not presently understood, but appears to be a property of the host cell.

GFAP expression in MHV-4-infected BALB/c and F_1 hybrid mouse astrocytes showing CPE was limited at 72 h postinfection. This is consistent with the lytic phase of infection and degradation of cellular components. This does not seem to be the case in infected SJL/J astrocytes, which show less CPE and continue to express GFAP as well as MHV-4 antigens (Fig. 4). Further studies will be necessary to determine the effect of chronic infection on GFAP expression.

The genetic resistance to MHV-4-induced fatal disease expressed by SJL/J mice in vivo is fully expressed in vitro at the level of the SJL/J neuron (8). The present study showed that nonneuronal cells from SJL/J mice (astrocytes) in vitro, in contrast to neuronal cells, can replicate MHV-4. Howver, compared with MHV-4-infected nonneuronal cells from susceptible BALB/c or F_1 hybrid mice, astrocytes from resistant SJL/J mice produced 10- to 50-fold less infectious virus. Thus, resistance to MHV-4 is

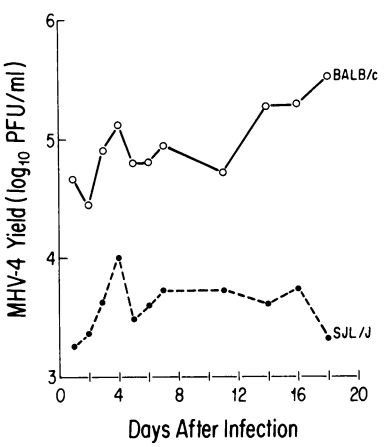

FIG. 3. Virus recovered at different times after infection from the supernatant fluids of BALB/c- and SJL/J-derived primary glial cell cultures infected with MHV-4 at a multiplicity of 0.1 at 14 days after seeding. The supernatant fluids were collected daily for the first 7 days and on days 11, 14, 16, and 18 after infection and assayed for infectious virus titer on L24-1 cells. Infected SJL/J-derived cultures produced 10- to 50-fold less infectious virus than did infected BALB/c-derived cultures.

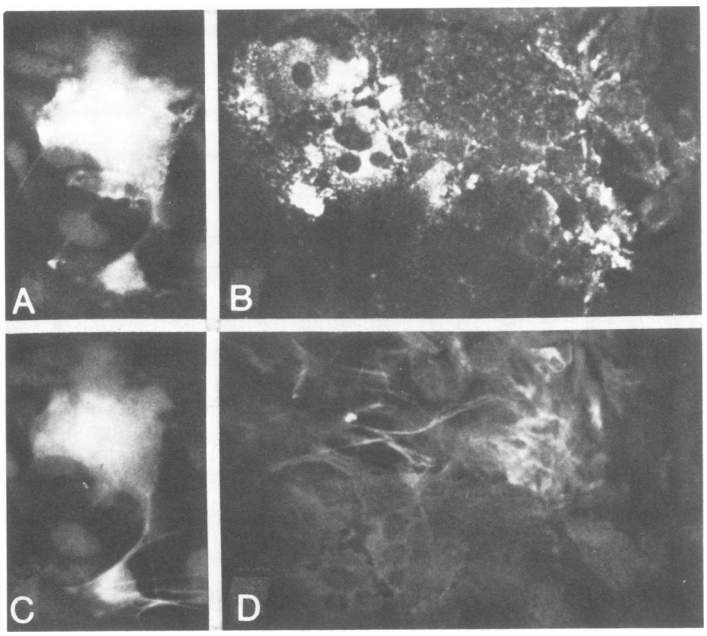

FIG. 4. Immunofluorescent staining of cells for MHV antigens and GFAP. MHV-infected SJL/J glial cell cultures had small foci of cells containing viral antigens (A). In contrast, MHV-4-infected BALB/c (not shown) and F_1 hybrid glial cells formed large foci, involving multiple cells, that contained viral antigens (B). GFAP was expressed in the same SJL/J cells that contained viral antigens (C), but its expression was markedly decreased in the BALB/c (not shown) and F_1 hybrid glial cells that contained viral antigens (D) (×400).

only partially expressed in SJL/J astrocytes compared with SJL/J neurons. A difference in response to MHV-4 infection was also obtained when comparing MHV-4 infection in primary BALB/c or F_1 hybrid glial cell cultures (limited lytic infection) with MHV-4 infection in primary neuron cultures (unlimited lytic infection, destroying the cultures within 5 days). Thus, susceptibility to MHV-4 infection is manifested differently in nonneuronal cells derived from BALB/c or F_1 hybrid mice than it is in their neurons. Differences in MHV-4 ultrastructural maturation nonneuronal cells compared with neuronal cells have recently been reported for another MHV-4-susceptible mouse strain (C57BL/6J) (4).

Genetically based differences in susceptibility to mouse hepatitis virus type 3 expressed at the cellular level were recently found in hepatocyte cultures derived from mouse strains both susceptible and resistant to it (1). Those results correspond to earlier findings showing that genetic resistance to MHV-4 is expressed at the level of the neuronal cell (8). The present study showed that despite the degree of resistance or susceptibility of the parental strain to MHV-4-induced disease, nonneuronal cells (astrocytes) in primary culture developed a limited lytic infection. This was manifested by a short lytic phase early in infection, followed by a less-lytic phase later during chronic infection. A similar shift between early and late infection was observed previously with MHV-4 infection in the RN-2 glial cell line (9). The occurrence of chronic infection in primary glial cell cultures derived from genetically susceptible and resistant mice allows the further study of the molecular mechanisms of host genetic influence on viral replication and conditions leading to the establishment of a chronic viral infection.

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