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Vulnerability of Rat and Mouse Brain Cells to Murine Hepatitis Virus (JHM-Strain): Studies In Vivo and In Vitro

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ABSTRACT The pathogenicity and cell tropism of mouse hepatitis virus (MHV-JHM-strain) in the developing mouse (Balb/c) and rat (Wistar and Lewis) brain were analysed. Intracranial infection of Balb/c mice at postnatal day 5 induced a lethal encephalitis in all animals. Of Wistar rats infected at day 2 or 5 after birth, 30 to 70%, respectively, survived. The distribution of viral antigen was studied in frozen brain sections of animals that died after infection; astrocytes were found to be the major virus-infected cell type throughout the central nervous system. More than 75% of the surviving rat pups developed paralysis, but viral antigen was detected in only few brain cells and not in astrocytes. The cell tropism of MHV-JHM was examined further in virus-infected glial cell cultures derived from brains of rats or mice. In the glial cultures derived from Wistar rats, only oligodendrocytes were infected, whereas in cultures derived from mouse or Lewis rat brain viral antigen was detected in both astrocytes and oligodendrocytes. Infection of astrocytes led to the formation of syncytia and degradation of the cytoskeleton. Infected rat oligodendrocytes gradually disappeared from the cultures because of cell death. These phenomena indicate that, besides an indirect autoimmune response triggered by infected astrocytes, direct virus-induced injury to astrocytes or to oligodendrocytes can have a dominant role in the neuropathogenicity of mouse hepatitis virus. The present results underscore the importance of species and developmental stage of experimental animals in the neurotropism and pathogenicity of MHV-JHM.

INTRODUCTION

Mouse hepatitis virus (MHV), a coronavirus, is widespread in mouse colonies. Although MHV causes no apparent illness in most (Fujiwara et al., 1976), an acute or chronic state of disease may result after experimental infection. The consequences of the infection depend on the pathogenic potential of the MHV strain (for review, see Wege et al., 1978) and on the mouse strain (Stohlman and Frelinger, 1978). Rats and hamsters are not natural hosts for MHV but are susceptible to experimental infection (Bailey et al., 1949; Cheever et al., 1949).

The neurotropic JHM strain of MHV can cause an acute encephalitis after intracranial (i.c.) inoculation of suckling rats. Postmortem analysis has shown that virus particles are present in oligodendroglial cells and in neurons (Wege et al., 1978). In contrast, a chronic

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demyelinating condition develops when weanling rats are infected. In this case, virions are found only in degenerated oligodendroglial cells (Wege et al., 1978; Nagashima et al., 1978). This shift in cellular tropism of MHV-JHM has also been described for infections of mice (Knobler et al., 1981a,b). It has been reported that a temperature-sensitive (ts) mutant of MHV is rarely lethal for mice but frequently results in chronic demyelination (Haspel et al., 1978). Virions were found mainly in oligodendrocytes (Knobler et al., 1982), suggesting that infection of this cell type by the mutant is the primary cause of demyelination.

Although replication of MHV-JHM in brain cells is well documented, cell tropism especially in rat brain, remains controversial. For example, in Wistar-Furth rats, only oligodendrocytes are target cells (Beushausen and Dales, 1985), whereas in Lewis rats all glial cells can be infected (Massa et al., 1986). In both cases the infection induces a pathological condition of the central nervous system (CNS). An age-related disease develops in infected Wistar-Furth rats (Sorensen et al., 1980), whereas an "experimental allergic encephalitis (EAE) type" mechanism of myelin destruction has been described in Lewis rats (Watanabe et al., 1983).

The present study examined the effects of MHV-JHM on cultured brain cells derived from Balb/c mice and from Wistar and from Lewis rats. The results obtained from studies in vitro were correlated with those of in vivo infections. The present observations indicate that sensitivity to MHV-JHM infection depends not only on the species of the animal, but is also affected by the differentiation of both oligodendrocytes and astrocytes.

MATERIALS AND METHODS

Cells

Mouse L-cells, obtained from Dr. E. Lehmann-Grube, Hamburg, West Germany, and Sac(−) cells, a Moloney sarcoma virus-transformed cell line defective in virus production (Weiland et al., 1978) were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (FCS), supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin (DMEM-10% FCS).

Cells from the cerebral hemispheres of newborn rats and mice were isolated as described previously (Van Berlo et al., 1986) and grown in DMEM-10% FCS supplemented with 10% heat-inactivated horse serum and 80 U/liter insulin. Cultures were grown for 7 days in a CO_2 incubator at 100% humidity and at 37°C and then were used for the infection experiments. At that stage, the cultures consisted predominantly of astrocytes as shown before (Van Berlo et al., 1986). Animals were obtained from the Central Institute for the Breeding of Laboratory Animals, TNO, Zeist, The Netherlands, and were tested serologically for the absence of anti-MHV antibodies by a plaque-reduction assay.

Glial cell cultures enriched in oligodendrocytes were prepared as described previously (Koper et al., 1986). Briefly, forebrains of 7-day-old rat pups were minced and incubated with trypsin at 37°C. The tissue was then gently disrupted by trituration in the presence of soybean trypsin inhibitor. The cell suspension was sieved through a fine nylon screen to remove tissue debris. Dissociated cells were suspended in DMEM containing 10% newborn-calf serum and plated onto poly-L-lysine-coated tissue culture dishes. The following day, the culture medium was replaced by a serum-free, chemically defined medium (Koper et al., 1986).

Viruses

The virus stock of MHV-JHM (Weiner, 1973) was plaque-purified twice and used to infect Sac(−) cells at a multiplicity of infection (MOI) of 0.001. Culture medium of infected cells was collected and centrifuged at low-speed centrifugation to remove cell debris. To precipitate the virus, 100g/liter polyethylene glycol 6000 (BDH, Poole, England) and 23.3 g NaCl/liter at pH 7.7 was added followed by incubation at 4°C for 2 hours and centrifugation (100,000 g, 15 minutes). The pellet was resuspended in a small volume of DMEM-3% FCS, and the suspension was then clarified by short centrifugation. To determine the yield of infectious virus, virus stocks were plaque-titrated on L-cells using an overlay of 1.5% Bacto-agar (Difco, Detroit, MI, USA) in DMEM-1% FCS. The plaques were read 2 days postinfection. Virus stocks usually had titers of about 10^9 plaque-forming units (PFU) per ml.

Inoculation of Animals

Wistar rats (2 and 5 days old) and Balb/c mice (5 days old) were injected i.c. with approximately 5×10^4 PFU of MHV-JHM in a volume of 10 μl. Control animals received 10 μl of the dilution medium.

Kinetics of Virus Growth

Sac(−) cells and primary brain cells were infected as described previously (Van Berlo et al., 1986). Culture supernatants of infected Sac(−) cells and brain cells were collected 10 and 26 hours postinfection (p.i.), respectively, and infectious virus was assayed by plaque titration.

Antisera and Indirect Immunofluorescence

A monoclonal antibody against the nucleocapsid protein of MHV-JHM (a gift from M. Buchmeier, La Jolla, CA, USA) and a rabbit anti-MHV-A59 serum (Rottier et al., 1981) were used to recognize the viral proteins of MHV-JHM. Rabbit antiglial fibrillary acidic protein (GFAP) serum (obtained from Dakopatts, Copenhagen, Denmark) and a monoclonal antigalactocerebroside (GalC) antibody (Ranscht et al., 1982) were used as markers for astrocytes (Bignami et al.,

1972) and oligodendrocytes (Raff et al., 1978), respectively. Monoclonal antibody A2B5 was used to distinguish between type 1 astrocytes, which are A2B5$^-$ and GFAP$^+$, and type 2 astrocytes (A2B5$^+$, GFAP$^+$) (Raff et al., 1983) and was also used to identify oligodendrocyte-type 2 astrocyte (O-2A) progenitor cells, which are A2B5$^+$, GalC$^-$, GFAP$^-$ (Raff et al., 1983).

Goat antimouse IgG3 or IgM, conjugated to rhodamine (TRITC), were used to visualize the binding of the anti-GalC antibody or the binding of the A2B5 antibody, respectively; and swine antirabbit immunoglobulin G (IgG) conjugated to fluorescein (FITC) was used to detect the binding of the anti-GFAP and anti-MHV antibodies. Double-immunolabelling procedures were carried out as described previously (Noble and Murray, 1984).

Studies In Vivo

Brains were quickly frozen in semisolid isopentane at −160°C. Sections (4 μm) were cut, fixed in methanol at −20°C for 10 minutes, and immunolabelled as described above.

Analysis of Virus-Specific Proteins in Infected Cells

Sac(−) and brain cells grown in 35-mm tissue culture dishes were infected and labelled from 7 to 9 hours p.i. (Sac[−]) or from 18 to 22 hours p.i. (brain cells) using 1 ml methionine-deficient minimal essential medium (MEM), supplemented with 5% FCS, and 25 μCi ^{35}S-methionine, 1230 Ci/mmol (The Radiochemical Centre, Amersham, England). After the labelling period, cells were rinsed with cold PBS and lysed for 5 minutes in 0.15 ml lysis buffer (Koolen et al., 1983). Lysates were centrifuged for 5 minutes at 10,000 g and then processed directly or stored at −70°C.

Virus-specific proteins were immunoprecipitated from 75 μl of portions of the lysates with 10 μl rabbit anti-MHV-JHM serum (a gift from S. Siddell, Wurtzburg, West Germany) following the methods previously described (Koolen et al., 1983). Samples were electrophoresed in 15% acrylamide-0.085% bisacrylamide gels (Rottier et al., 1981), and proteins were visualized by fluorography on preflashed Fuji RX film at −70°C.

RESULTS

MHV-JHM Infection In Vivo

The mortality of rat pups after viral infection dropped rapidly with the age of the animals. Whereas 70% (7/10) of the rat pups died of acute encephalitis when infected at postnatal day 2, only 31% (8/26) died when infected 5 days after birth. In addition, most (14/18) of the rat pups inoculated i.c. with MHV-JHM at postnatal day 5 that survived developed tremors and paralysis of the hind legs. In contrast to the effects of MHV-JHM inoculation in rats, mice infected with MHV-JHM at 5 days after birth all died (Table 1).

To determine the presence of viral antigen in the CNS of acutely or lethally afflicted and chronically paralysed rats, frozen brain sections were immunolabelled with viral-specific antibodies and anti-GFAP antibodies and examined by fluorescence microscopy. In the acute disease, high concentrations of viral antigen were found throughout the brain. Double immunolabelling showed that viral antigen could be traced in astrocytes (Fig. 1A,B) but was also present in other cells. In contrast, the very low levels of viral antigen present in brain sections of paralysed animals were not present in astrocytes (Fig. 1C,D). No antigen was detected in the brains of uninfected animals (Fig. 1E,F).

MHV-JHM Infection of Glial Cells in Primary Culture

To study the infection of astrocytes with MHV-JHM in vitro, cultures of brain cells derived from either newborn mice or rats were prepared and infected with virus. Twenty hours after infection, cultures, consisting predominantly of type 1 astrocytes (Van Berlo et al., 1986), were fixed and immunolabelled with anti-MHV and anti-GFAP antibodies. Many multinucleated cells (syncytia) were observed in cultures of mouse brain cells, and these syncytia were positive both for viral antigens and GFAP (data not shown). Syncytium formation was associated with a breakdown of glial filaments, as judged by their diffuse distribution within the infected cells (Fig. 2A). In contrast, no MHV-infected astrocytes and no multinucleated GFAP-positive cells were observed in cultures derived from the brains of newborn rats (Fig. 2B).

To analyse the expression of viral proteins, infected Sac(−) and mouse or rat astrocytes were labelled with ^{35}S-methionine. Viral proteins in the lysates of these infected cells were immunoprecipitated with a serum, directed against purified virions, and separated by polyacrylamide gel electrophoresis (PAGE). In MHV-JHM infected Sac(−) cells, eight virus-specific ^{35}S-methionine labelled polypeptides were detected (Fig. 3). The four low-molecular-weight proteins (23,5K, 21,5K, 20,5K, and 18K) are molecular species of the E1 protein

TABLE 1. Paralysis and death of Wistar rats and Balb(c) mice after inoculation with MHV-JHM

Postnatal age at time of inoculation[a] (days)	Total number	Number paralysed (%)	Number dead (%)
Rats			
2	10	0 (0)	7 (70)
5	26	14 (54)	8 (31)
Mice			
5	20	0 (0)	20 (100)

[a]Each rat or mouse was inoculated (i.c.) with 5×10^4 PFU in 0.01 ml.

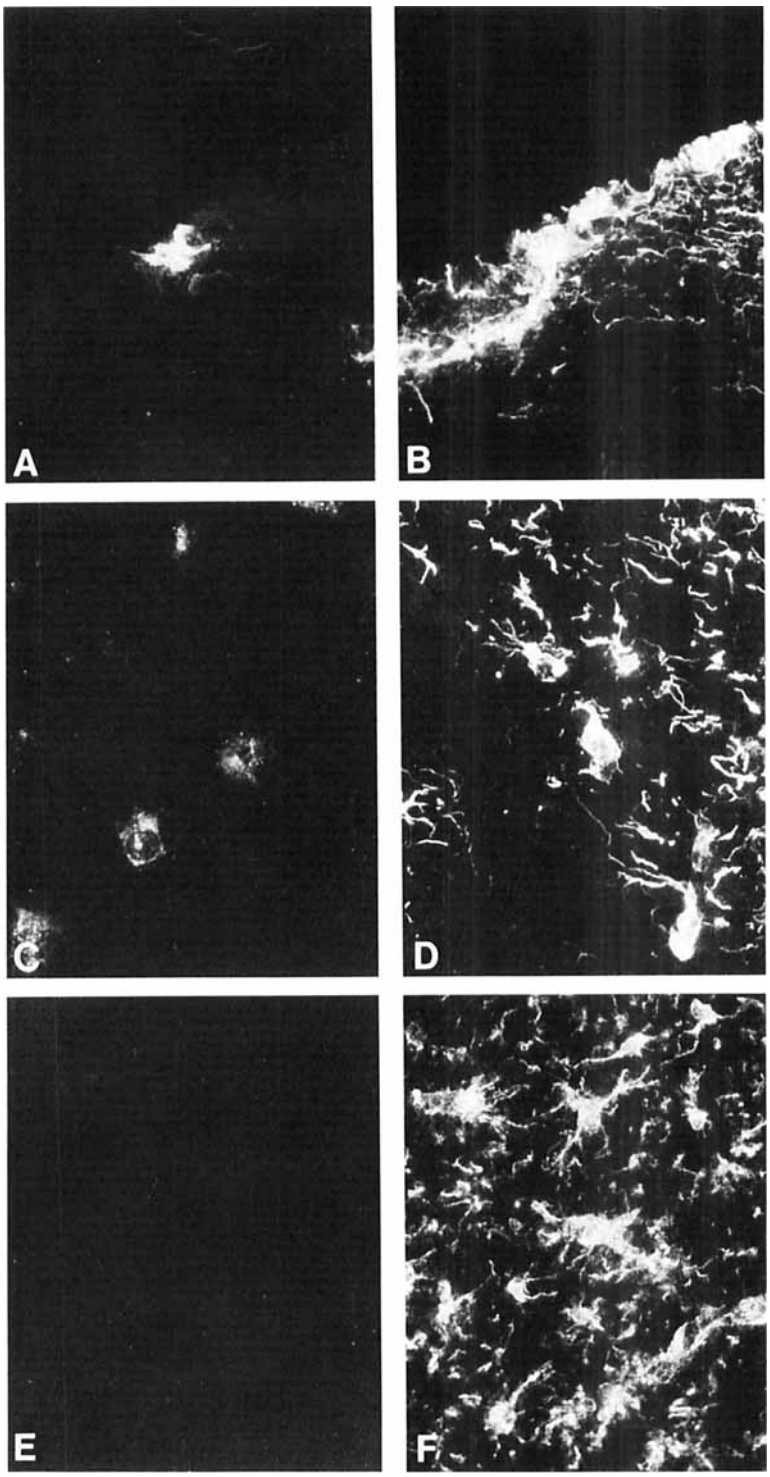

Fig. 1. Localization of viral antigen in brain sections of MHV-JHM-infected rats. Double-label immunofluorescence was performed on frozen brain sections from Wistar rats. **A,B:** Rats that were acutely and lethally affected. **C,D:** Rats chronically paralysed. **E,F:** Mock-infected rats. Viral antigen was detected by a monoclonal antibody against the viral nucleocapsid protein and visualised by a TRITC-conjugated goat antimouse IgG (A, C, E). Sections were also stained for GFAP with a rabbit antiserum and a FITC-conjugated swine antirabbit IgG (B, D, F). ×240.

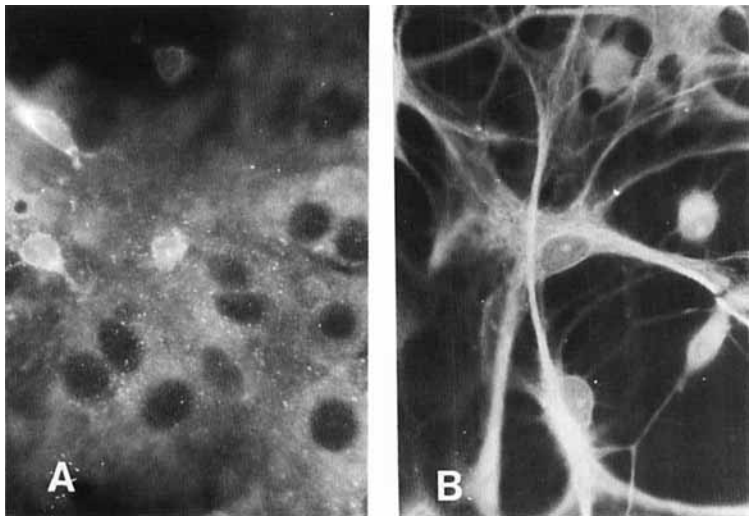

Fig. 2. Effect of MHV-JHM infection on cultured astrocytes from the mouse and rat. **A:** Brain cells derived from newborn Balb/c mice. **B:** Brain cells from Wistar rats after growth in culture for 7 days. Cells were inoculated with MHV-JHM (MOI 30) and 24 hours later fixed and stained for GFAP using a rabbit antiserum and a FITC-conjugated swine antirabbit IgG. Note that syncytium formation with breakdown of glial filaments is observed only in the mouse preparation (A). ×320.

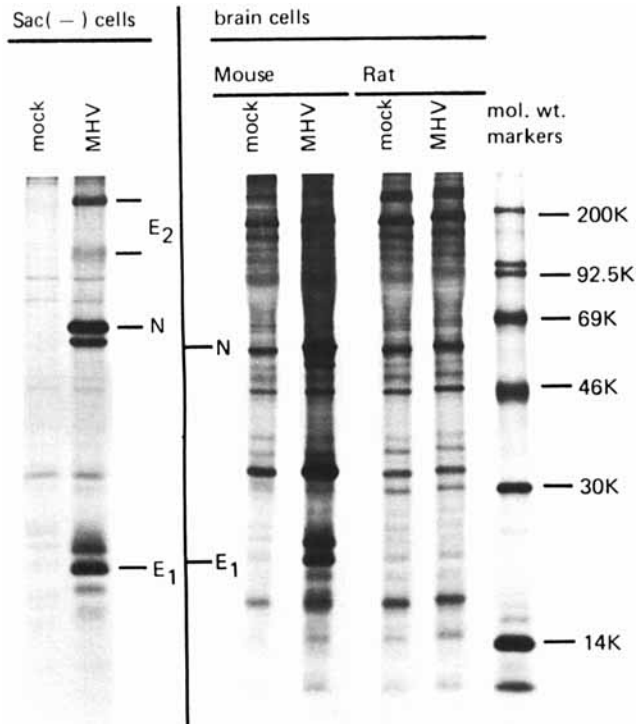

Fig. 3. Electrophoretic analysis of viral proteins in MHV-JHM infected cells. Sac(−) cells, Wistar rat, or mouse astrocytes were infected with MHV-JHM (see legend to Fig. 2) and incubated with ^{35}S-methionine from 7 to 9 hours (Sac[−] cells) or from 18 to 22 hours p.i. (astrocytes). Cells were harvested and viral proteins immunoprecipitated from cell lysates using an anti-MHV-JHM serum. Immunoprecipitates were analysed by PAGE in 15% polyacrylamide gels. Molecular weight of standards (10^{-3}) and MHV proteins E1, E2, and N are indicated.

(Siddell et al., 1981). The high-molecular-weight protein bands of 90K and 160K are the membrane protein (E2) and its precursor, respectively (Siddell et al., 1981). In addition, two prominent bands were observed with molecular weights (60K and 57K) in the range of the nucleocapsid protein (N). The lower band might be a degradation or not-phosphorylated product of the upper band. The induced polypeptides in MHV-JHM infected mouse brain cells were comparable to those in infected Sac(−) cells. In rat astrocytes, no viral-induced proteins were observed (Fig. 3).

Additional experiments were carried out whether or not the cultures produced infectious virus. Culture supernatants were plaque-titrated on L-cells, and the amounts of infectious virus particles in the mouse and rat brain cell cultures were compared with those in infected Sac(−) cells. The results showed that the JHM strain of MHV was produced by Sac(−) cells (150 PFU/cell at 10 hours p.i.) and by cultures of mouse brain cells (85 PFU/cell at 26 hours p.i.), but not by cell cultures derived from rat brain.

MHV-JHM Infection of Rat Oligodendrocytes in Culture

To find out whether or not rat oligodendrocytes were susceptible to infection with MHV-JHM, cells isolated from the forebrains of 7-day-old Wistar rats were grown in a serum-free, chemically defined medium that favours the development of oligodendrocytes (Koper et al., 1984). One day after plating, most of these cells were positive for A2B5 and expressed neither GalC nor

GFAP. This antigenic phenotype is characteristic of the oligodendrocyte type 2 astrocyte progenitor cells described by Raff et al. (1983). As expected, after 6 days of growth in a chemically defined medium, most A2B5-positive cells had differentiated into GalC-positive oligodendrocytes, and virtually all A2B5-positive cells had disappeared from the cultures (Van der Pal et al., 1988).

Infection of these oligodendrocyte-enriched cultures with MHV-JHM showed that 1 day after infection only a few GalC-positive (Fig. 4A,B) and no A2B5-positive cells (results not shown) contained viral proteins. After 36 hours infected oligodendrocytes lost their complex network of branches and appeared to be dying (Fig. 4C,D).

Similar experiments were performed with oligodendrocyte-enriched cultures derived from Lewis rats or Balb/c mice. Double-label immunofluorescence revealed that oligodendrocytes of both species stained strongly with the anti-MHV antibody (Fig. 5A,C, respectively). Cultures derived from Lewis rats also showed foci of

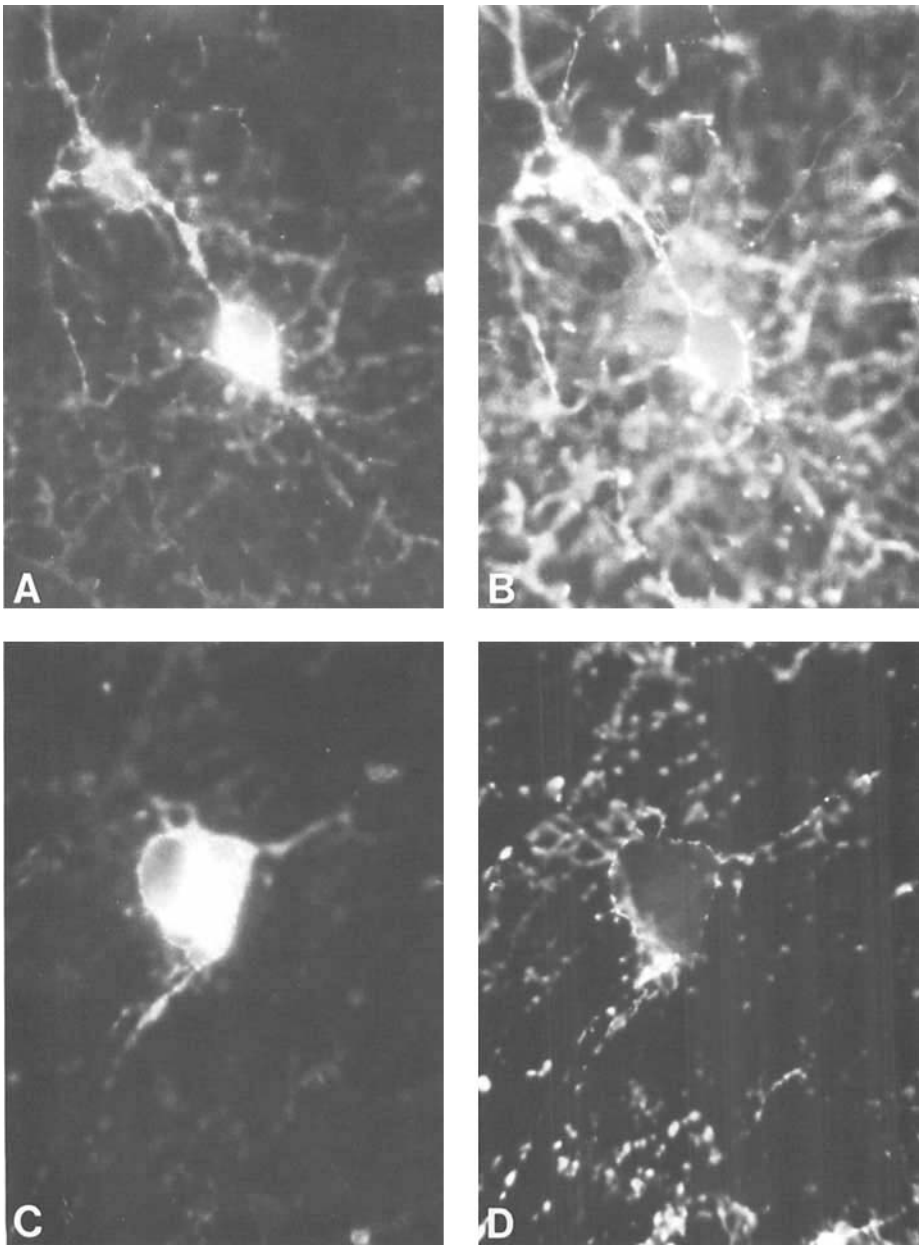

Fig. 4. Rat oligodendrocytes in culture can be infected with MHV-JHM. Oligodendrocyte-enriched cultures derived from cerebra of 1-week-old Wistar rats were infected with MHV-JHM on day 6 in culture. One day (**A,B**) or 36 hours (**C,D**) later, the preparation was incubated with a monoclonal antibody against GalC, followed by a TRITC-conjugated goat antimouse IgG. After fixation the preparation was incubated with a rabbit anti-MHV antiserum coupled with FITC. A,C: FITC fluorescence (MHV^+); B,D: TRITC fluorescence ($GalC^+$). Note the necrotic appearance of an infected oligodendrocyte in panels C and D. ×380.

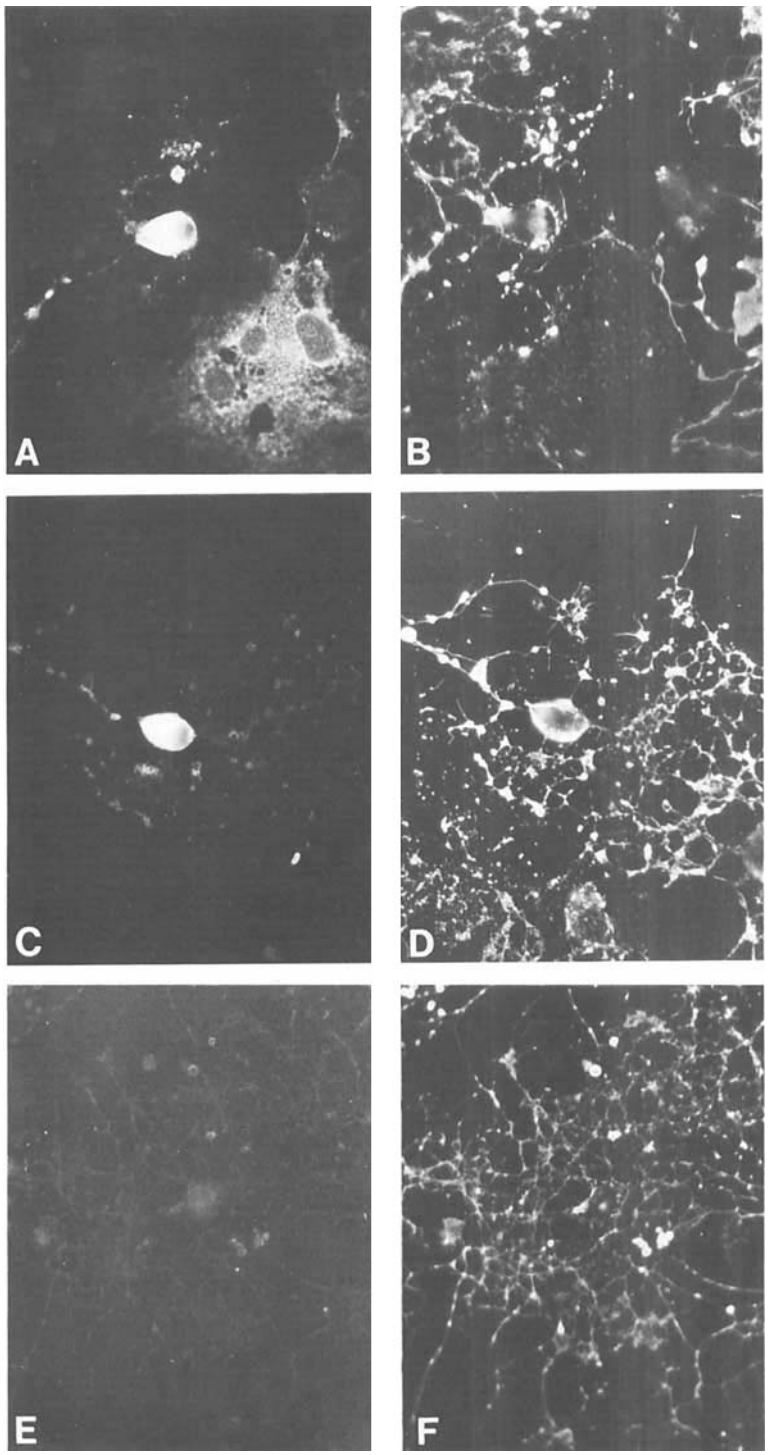

Fig. 5. Rat and mouse oligodendrocytes after MHV-JHM infection. Oligodendrocyte-enriched cultures derived from cerebra of 1-week-old Lewis rats (**A, B**) or Balb/c mice (**C, D**) were infected with MHV-JHM on day 6 in culture. Controls of mock-infected cultures (rat) are shown in panels **E** and **F**. After 24 hours; virus-positive cells (A,C,E) and GalC-positive oligodendrocytes (B,D,F) were visualized as described in the legend of Figure 4. Note the enlarged, virus-positive syncytia in panel A. ×240.

virus-positive GalC-negative multinucleated cells, probably infected astrocytes (Fig. 2). In primary brain cell cultures from Lewis rats consisting mainly of astrocytes, many multinucleated cells were observed that were positive for viral antigen (results not shown). No viral antigen was detected in mock-infected cells (Fig. 5E,F).

It should be emphasized, however, that in cultures from both rat species, few GalC-positive cells were infected. The number of infected oligodendrocytes increased with the virus dose when cultures were inoculated at higher multiplicities of infection (MOI) (results not shown). However, even after an infection with an MOI of 100 FPU/cell, only a small fraction (less than 10%) of the $GalC^+$ oligodendrocytes was infected compared with mouse cultures (Table 2). In addition, the number of infected oligodendrocytes in Wistar rat cultures decreased significantly after 72 hours, suggesting that the infection was lethal for these cells. In Lewis rats the number of infected cells remained constant, whereas in Balb/c mice there was an increase in the number of viral positive cells, suggesting that the produced virus infected other cells.

DISCUSSION

The effects of MHV-JHM infection in the CNS depend on the species of the experimental animal. Mice developed an acute, lethal encephalitis within 3 to 7 days; in contrast, rats that survived an infection by MHV-JHM often exhibited a chronic disease (Table 1).

How can we explain these differences between related animal species? An answer might be that the susceptibility to infection with MHV of the various CNS cell types is species dependent. The present experiments support this hypothesis and agree with previous observations made in situ (Powell and Lampert, 1975; Nagashima et al., 1978; Sorensen et al., 1980) and in vitro (Dubois-Dalcq et al., 1982; Beushausen and Dales, 1985; Wilson et al., 1986). When MHV-JHM infection caused an acute encephalitis in young rat pups, antigen could be found throughout the brain, mainly in astrocytes (Fig. 1A,B). However, when chronic paralysis followed infection, unidentified cells were found to be positive for the viral antigen. These results suggest that a susceptibility of immature brain cells to infection by MHV-JHM may result in an acute disease in young rat pups. Acute encephalitis in mice, after infection with MHV-JHM, is always associated with infection of astrocytes (Wilson et al., 1986). This susceptibility to MHV-JHM infection is found also in cultures of mouse astrocytes and Lewis rats (Massa et al., 1986). Astrocytes from Wistar rats are not susceptible to MHV-JHM infection, suggesting that they lack distinct binding sites for MHV-JHM. In this context, it is noteworthy that Boyle et al. (1987) reported that target cells from susceptible animals have a MHV-binding molecule on their membranes. A specific receptor, therefore, must be present on astrocytes from Lewis rats, as viral replication occurs in these cells.

TABLE 2. Number of virus-replicating mouse or rat oligodendrocytes in culture[a]

Hours after inoculation	Number of virus-positive cells[b]		
	Rats		Mice
	Wistar	Lewis	Balb/c
24	120	58	616
72	16	62	1132

[a]As determined by MHV-JHM/GalC double-label immunofluorescence.
[b]Per 5×10^3 oligodendrocytes.

In cultures of Wistar rat glial cells, only oligodendrocytes (GalC-positive) were infected (Fig. 4), whereas A2B5-positive cells (progenitor cells and type-2 astrocytes) were resistant to virus infection. Beushausen and Dales (1985) showed that MHV-JHM replication in oligodendrocytes, derived from newborn Wistar-Furth rats, is maximal between 10 and 15 days after explantation. Replication is suppressed between day 15 and 21, which fits with the report of the same group (Sorensen et al., 1980) that their rats became highly resistant to MHV-JHM infection 3 weeks after birth. It appears, therefore, that MHV-JHM can induce paralysis in Wistar rats only during a short time span of the differentiation phase of oligodendrocytes. Oligodendrocytes (Walker et al., 1984) and astrocytes (Schachner, 1982) in culture develop according to a fixed time schedule. Future studies are necessary to sort out which differentiation phase and surface property is essential for infection of oligodendrocytes by MHV-JHM.

The present results underscore the notion that pathogenesis of MHV-JHM coronavirus-induced demyelination may involve two completely different mechanisms. First, astrocytes of Lewis rats infected with MHV-JHM support a persistent infection in which the rate of virus production is low (Massa et al. 1986). Such infections could initiate processes having indirect long-term effects on oligodendrocytes and myelin. Second, an infection by MHV-JHM in astrocytes in mice (Wilson et al., 1986) and in Wistar rat pups by MHV-JHM causes a fatal acute disease. Older Wistar rat pups survive but sometimes become paralysed; lytic infection of oligodendrocytes may cause foci of viral induced demyelination.

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