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Selective Tropism of a Neurotropic Coronavirus for Ependymal Cells, Neurons, and Meningeal Cells

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The ability of a neurotropic virus, mouse hepatitis virus type 3 (MHV3), to invade the central nervous system (CNS) and to recognize cells selectively within the brain was investigated in vivo and in vitro. In vivo, MHV3 induced in C3H mice a genetically controlled infection of meningeal cells, ependymal cells, and neurons. In vitro, purified MHV3 bound to the surface of isolated ependymal cells and cultured cortical neurons but not to oligodendrocytes or cultured astrocytes. MHV3 replicated within cultured cortical neurons and neuroblastoma cells (NIE 115); infected cultured neurons nonetheless survived and matured normally for a 7-day period postinfection. On the other hand, MHV3 had a low affinity for cortical glial cells or glioma cells (C6 line), both of which appear to be morphologically unaltered by viral infection. Finally, MHV3 infected and disrupted cultured meningeal cells. This suggests that differences in the affinity of cells for MHV3 are determinants of the selective vulnerability of cellular subpopulations within the CNS. In vivo, a higher titer of virus was needed for CNS penetration in the genetically resistant (A/Jx) mice than in the susceptible (C57/BL6) mouse strain. However, in spite of viral invasion, no neuropathological lesions developed. In vitro viral binding to adult ependymal cells of susceptible and resistant strains of mice was identical. Genetic resistance to MHV3-CNS infection appeared to be mediated both by a peripheral mechanism limiting viral penetration into the CNS and by intra-CNS mechanisms, presumably at a stage after viral attachment to target cells.

A genetically controlled, persistent central nervous system (CNS) infection is induced in C3H mice inoculated with mouse hepatitis virus type 3 (MHV3), a member of the coronavirus group (14, 21, 23). Intraperitoneal (i.p.) injection of MHV3 into adult C3H mice results either in early death due to hepatitis or in the development of a chronic disease with neurologic manifestations and virus persistence in surviving animals. Chronic neurologic disease results from transient meningitis, ependymitis, and encephalitis, beginning 3 to 4 weeks after infection and followed by a permanent communicating hydrocephalus. Later (after 6 weeks postinfection), a chronic thrombotic vasculitis develops affecting meningeal and parenchymal vessels at the brain stem level (21, 23). Some strains (e.g., A/Jx) of mice are resistant to the virus, while others (C57/BL6 mice) are highly susceptible and without exception develop acute hepatic necrosis leading to death within a few days of injection (13).

The presence of a receptor for a given virus on the cell membrane is a primary factor in determining the affinity of that virus for the cells and appears to contribute to the pathogenicity of viral disease (5, 10, 20). Studies of viral binding to isolated subpopulations of CNS cells provide an in vitro approach to the study of viral tropism in the nervous system, and patterns of viral binding in vitro have been found to correlate with the pathologic consequences of viral infection in vivo (6, 20). The aim of the present work is to define the selective affinity of MHV3 for CNS cells and to approach the question of the genetic control of MHV3 infection.

MATERIAL AND METHODS

Mice. Inbred mouse strains C3H/He Orl, A/Jx, and C57/BL6 were purchased from the Centre de Sélection et d'Élevage d'Animaux de Laboratoire, Centre National de la Recherche Scientifique, Orléans-La Source, France. Animals were infected i.p. with different doses of virus. At different times after infection, animals were anesthetizied with ether and perfused with phosphate-buffered saline (PBS) for 5 min through the left ventricle. The organs were removed, added to 2 ml of PBS solution, frozen at −70°C, thawed, sonicated, and assayed for the presence of virus.

Virus production, purification, and titration. MHV3 was originally obtained from Jean-Marie Dupuy, Bicêtre, France, and was propagated in C57/BL6 mice. The virus was plaque purified in L cells. To produce stock virus, we inoculated L-cell monolayers with MHV3 at a multiplicity of infection of 0.1 to 1 PFU per cell. After an adsorption period of 1 h at 37°C, culture medium (Eagle minimal essential medium, 5% fetal calf serum, 1% of a stock solution of 10,000 U of penicillin per ml–10,000 μg of streptomycin per ml, 1% of a stock solution of 200 mM glutamine) was added, and cells were incubated at 37°C in a 5% CO_2 atmosphere. Released virus was harvested 24 h after inoculation. Preliminary experiments demonstrated that at this time the highest virus yields (10^5 to 10^6 PFU/ml) and cell fusions were observed.

Virus purification was performed according to the technique of Sturman et al. (17). Briefly, 400 ml of supernatant was collected, and the virus was precipitated by polyethylene glycol. The precipitate was collected by centrifugation at 10,000 × *g* for 30 min at 4°C, suspended in 3 ml of TMEM buffer (0.05 M Tris-maleate, 0.001 M EDTA, 0.1 M NaCl [pH 6]), and layered over a discontinuous gradient of 3 ml each of 30 and 50% (wt/wt) sucrose in TMEM buffer. After centrifugation for 4 h at 30,000 rpm in a Spinco SW41 rotor, the virus band at the 30 to 50% interface was collected, dialyzed against TMEM buffer, divided into aliquots, and frozen at −70°C. Titers of purified virus ranged between 5 × 10^6 and 10^7 PFU/ml.

Virus titers were determined by plaque titration with L cells as described by Sturman and Takemoto (18) or, for very

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TABLE 1. Induction of acute hepatitis and chronic CNS disease in C3H mice inoculated with MHV3

Age of infected mice (wk)	No. of mice inoculated	Dose (PFU) of virus[a]	% of mice dead after acute illness[b]	% of mice with chronic disease[c]
12–14	26	3×10^2	15	86
9	30	3×10^2	66	100
12	21	3×10^3	33	93

[a] MHV3 was injected i.p. in 0.1 ml of medium.
[b] As judged on day 10 p.i. Death generally occurred between days 3 and 5 p.i.
[c] As judged clinically during month 2 p.i. Percentages are based on the number of animals surviving after day 10 p.i.

low virus titers, by the 50% lethal dose technique. The 50% lethal dose in C57/BL6 mice was determined to be 1 to 10 PFU.

Isolation of ependymal cells and oligodendrocytes. Ependymal cells were isolated from adult mice brains as previously described (22). Isolated ependymal cells were identified by the presence of cilia as viewed under light microscopy.

Oligodendrocytes were isolated by the technique of Snyder et al. (16). Cells were defined as oligodendrocytes by their ability to be stained by rabbit anti-galactocerebroside antibody and fluorescein isothiocyanate (FITC) goat anti-rabbit immunoglobulin.

Cortical cell culture. Mouse cortical neuronal cell cultures were established from 15-day-old C3H embryos by the technique of Swaiman et al. (19). Briefly, cells were dissociated by trypsination (0.25% trypsin [GIBCO Laboratories, Grand Island, N.Y.], 30 min, 37°C). Approximately 7×10^5 cells were cultured in Eagle minimal essential medium modified to include 6 g of glucose per liter and 3.7 g of $NaHCO_3$ per liter with 10% fetal calf serum and 1% glutamine on a collagen-coated 16-mm well (Nunc, Roskilde, Denmark) at 37°C in a 10% CO_2 atmosphere. An antimitotic agent (5-fluoro-2'-deoxyuridine [10 μg/ml], uridine [25 μg/ml]) was added to the medium between days 5 and 6 of the culture. After 2 to 5 days in vitro, the cultures were predominantly made up of young neurons, with a small percentage of glial precursors. By week 2 in vitro, the cultures consisted of a monolayer of glial cells and other nonneuronal cells (mesenchymal cells and fibroblasts), above which were numerous mature neurons. After week 4 in vitro, mature neurons were less frequently observed, and by week 5 in vitro, cultures consisted predominantly of a monolayer of glial cells and other nonneuronal cells. In the present study, cells were classified as neurons based upon their appearance under phase-contrast microscopy and by positive tetanus toxoid staining or anti-A2B5 antibody (mouse immunoglobulin G monoclonal antibody against neuronal cell surface antigen [Sera Laboratory, diluted 1/100) or anti N-Cam antibody (diluted 1/2, kindly given by A. Goffinet, Brussels, Belgium [8]). Cells were classified as astrocytes by positive labeling with antibody to glial fibrillary acidic protein (GFAP) (tetanus toxoid antibody, anti-tetanus toxoid antibody, and anti-GFAP antibody as previously described were generously given by M. A. Dichter) [4]).

Mouse cortical glial cells cultures were prepared from the cortices of C3H newborn mice and plated with an inoculum of 10^5 cells per 16-mm uncoated well (or for virus titration experiments, 35-mm multidish plates [Nunc]). The subsequent preparation and maintenance of these cultures were similar to those of neuronal glial cultures, except that the medium was initially changed after 24 and 48 h and 3 to 4 days thereafter (15). The percentage of fibroblasts (as judged by indirect immunofluorescence staining with anti-fibronectin monoclonal antibody [Sera Lab, clone 2.3F9]) varied between 1% (day 5 postplating) and 10% (day 12 postplating).

Mixed neuronal-glial cultures or glial cultures of the same postconceptional age were exposed at different times postplating to 5×10^2 PFU of MHV3 (multiplicity of infection = 10^{-4} PFU per cell) for 1 h at 37°C, followed by removal of the supernatant and the addition of fresh complete medium. Morphological alterations of cells were monitored every day for 20 days, and 200 μl of supernatant was removed daily for viral titration.

Immunofluorescence staining. Isolated cells (5×10^5 from either the ependymal cell or the oligodendrocyte-enriched cell suspension were pelleted and incubated with 50 μl of purified MHV3 (10^7 PFU/ml) for 30 min at room temperature and washed twice. Viral binding was revealed by indirect immunofluorescence with an anti-MHV3 antibody consisting of pooled sera (from chronically infected C3H mice), diluted 1:40 (initial titer, 1:6,400 by enzyme-linked immunosorbent assay) and absorbed against normal brain tissue for 1 h at room temperature. Cells were incubated with anti-MHV3 antibody for 20 min at 4°C, washed twice, and stained with FITC-goat anti-mouse immunoglobulin antiserum (FITC-Gamig) at 4°C for 20 min, washed again, fixed in 1% paraformaldehyde, and observed under a Zeiss fluorescence microscope with epi-illumination. For double-labeling experiments, at the end of the preceding procedure, cells were incubated successively with rabbit anti-galactocerebroside antibody (30 min at 4°C) and rhodamin-conjugated goat anti-rabbit antibody (30 min at 4°C).

For a demonstration of viral binding to the surface of glial cells and neurons, appropriate cultures were grown on glass cover slips or on tissue culture chamber slides (Miles Scientific, Div. Miles Laboratories, Inc., Naperville, Ill.), washed

TABLE 2. Viral titers in livers and brains of mice from different genetic origin inoculated i.p. with MHV3 at day 4 p.i.

Injected dose (PFU/animal) of virus	Organ	Viral titers (PFU/g) in organs from mouse strain[a]: C3H	A/Jx	C57/BL6
10^2	Liver	$2 \times 10^4 \pm 2.2$	$1.3 \times 10^5 \pm 2.3$	$7.9 \times 10^5 \pm 4.7$
	Brain	$1 \times 10^3 \pm 1.5$	$<5 \times 10^2$[b]	$2.1 \times 10^3 \pm 1.4$
10^4	Liver	$2.1 \times 10^5 \pm 2.2$	$6.3 \times 10^5 \pm 7.2$	$2.3 \times 10^6 \pm 1.7$[c]
	Brain	$1.6 \times 10^3 \pm 1$	$2.6 \times 10^4 \pm 4.3$	$1.2 \times 10^3 \pm 0.7$

[a] Results are expressed as the mean ± 1 standard deviation of five to eight experiments. The rates of viral replication were similar in the three strains of mice as measured at days 1, 2, 3, 4, 5, and 7 p.i. All rates peaked at day 4 p.i.
[b] Titers were lower than the threshold of viral detection in tissue.
[c] Titers on day 3 p.i., just before death.

TABLE 3. Affinity of purified MHV3 for isolated ependymal cells and oligodendrocytes as indicated by indirect immunofluorescence

Enriched suspension	Incubation with MHV3[a]	% fluorescent cells (mean ± 1 SD)
Ependymal cells[b]	−	7.6 ± 4.6
	+	82.3 ± 3.7
Oligodendrocytes[c]	−	11.2 ± 5.6
	+	25.3 ± 9.6

[a] −, No virus; +, presence of virus.
[b] Percentage of fluorescent cells among ciliated cells. Mean ± standard deviation of five different experiments.
[c] Percentage of fluorescent cells among round nonciliated cells (see text). Mean ± standard deviation of five different experiments.

in PBS, overlaid with 50 μl of purified MHV3 (10^7 PFU/ml) at room temperature for 30 min, washed twice, and incubated with anti-MHV3 antibody and FITC-Gamig as described above. Cultures were then fixed with 10% paraformaldehyde (30 min) and cold ethanol with 5% acetic acid (10 min).

To detect the presence of viral antigen in infected neurons, we exposed cultures on tissue culture chamber slides (Miles) to 10^2 PFU of MHV3 at 37°C for 1 h, followed by the removal of the supernatant, addition of complete media, and examination after 24 or 48 h. Cells were washed in PBS, fixed as above, and treated with anti-MHV3 antibody or normal mouse serum as a negative control and with FITC-Gamig. When double-labeling experiments were performed, cells were subsequently treated with anti-GFAP rabbit antibody (30 min at 4°C) and rhodamin-conjugated goat anti-rabbit antibody (30 min at 4°C). In one series of experiments, rabbit anti-MHV3 antibody (Microbiological Associates, Bethesda, Md.; catalog no. 30-121 N) and rhodamin-conjugated goat anti-rabbit immunoglobulin were used, followed by anti N-Cam or anti-A2B5 antibody and fluorescein-conjugated goat anti-mouse immunoglobulin; incubations were overnight at 4°C.

Viral replication in continuous neuronal and glial cells lines. C6 glial cells and a neuroblastoma cell line (NIE 115) were maintained in culture in the same medium as cortical cell cultures and were similarly infected with MHV3. Morphological analysis of infected cells, as well as viral titration of the supernatant, was then performed.

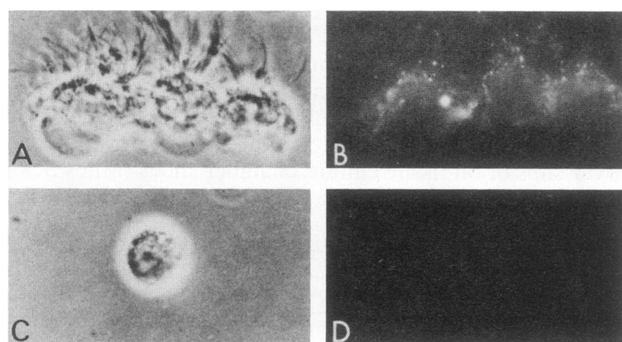


FIG. 1. Binding of MHV3 to isolated ependymal cells or oligodendrocytes from adult C3H mice demonstrated by indirect immunofluorescence. (A) Unstained ependymal cells examined by phase microscopy; (B) the same field seen by fluorescence microscopy showing surface labeling of the ependymal cells after incubation with MHV3; (C) unstained oligodendrocytes examined by phase microscopy; (D) the same field as in panel C seen by fluorescence as performed for panel B (see Materials and Methods). Magnification, ×400).

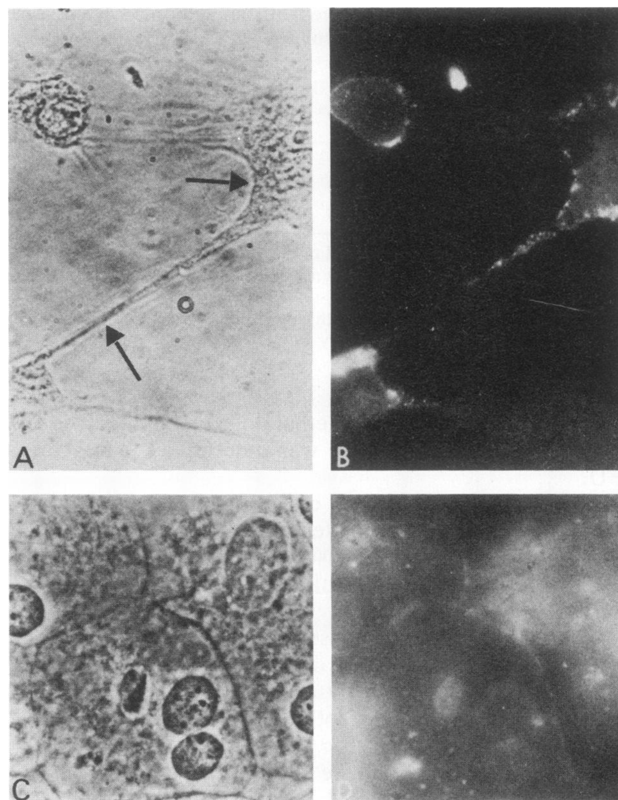


FIG. 2. Binding of MHV3 to cultured neurons and astrocytes demonstrated by indirect immunofluorescence. Cortical cell cultures were obtained from 15-day-old embryos and studied for 9 days after plating. Viral binding was revealed by indirect immunofluorescence. Shown are Phase-contrast micrographs of cultures containing neurons (A, arrows) with their characteristic processes lying over flat astrocytes (C) and the same fields seen by fluorescence microscopy displaying surface labeling of neurons (B) but no staining of background nonneuronal cells (D). Magnification, ×400).

Meningeal cell cultures. Meninges from newborn mice were dissected under a microscope, and 1-mm explants were cultured in 25-cm² plastic tissue culture flasks (T25; Nunc) with Eagle minimal essential medium containing nonessential amino acids, 10% fetal calf serum, 1% penicillin-streptomycin, and 1% glutamin. Cells were tested at transfer 3. More than 90% of the cells were positive for fibronectin antigen and negative for GFAP.

RESULTS

In vivo induction of persistent CNS infection with MHV3. Induction of acute hepatitis or chronic CNS disease was dependent upon both the age of recipient mice and the injected dose of virus (Table 1) (14).

Viral titers in the brain of MHV3-infected C3H mice (10^2 PFU per animal) reached 10^3 PFU/g between days 3 and 4 postinfection (p.i.) (Table 2). After day 4 p.i., viral titers decreased to a low but persistent level (between 10 and 100 PFU/g). This viral titer was barely detectable by plaque assay, but viral persistence was clearly demonstrated as late as day 112 p.i. by the ability of brain extract from chronically infected animals to induce acute lethal hepatitis in suscepti-

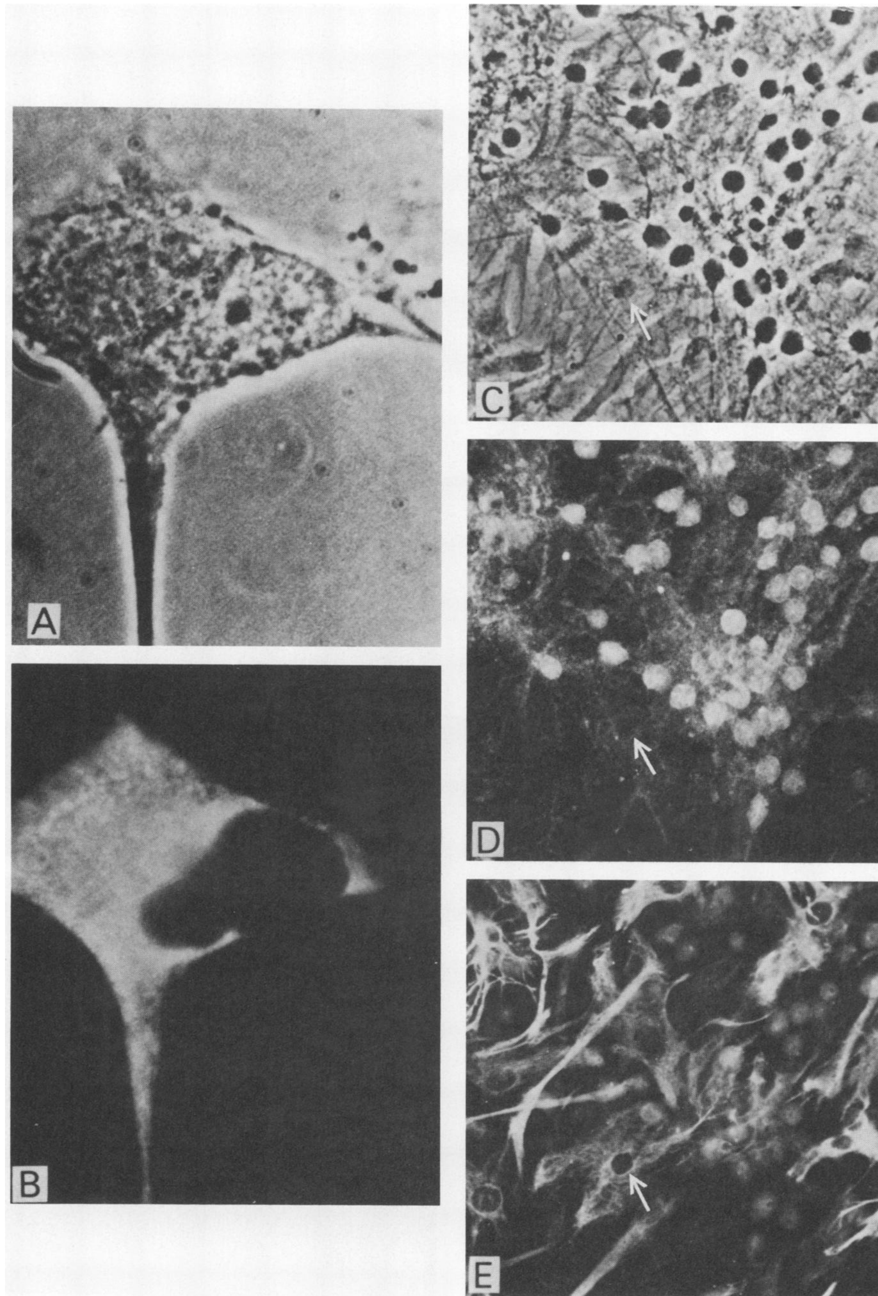


FIG. 3. Immunofluorescent localization of MHV3 antigens in MHV3-infected fetal cortical cell cultures. Cortical cell cultures were obtained from 15-day-old embryos infected with MHV3 (5×10^2 PFU per well) 9 days after plating and examined after 48 h. Cells were fixed and treated with anti-MHV3 antibody and FITC-Gamig. Shown is a large neuron seen under a phase microscope (A) and with intense intracytoplasmic labeling revealed by fluorescence microscopy (B). Magnification, ×760). A similarly MHV3-infected embryonic cortical cell culture was treated with anti-MHV3 antibody and FITC-Gamig, followed by anti-GFAP antibody and rhodamin-conjugated Gamig. Shown are the culture as seen under a phase microscope (C) and fluorescence-positive cells (MHV3 antigen [D]), which are distinct from rhodamin-positive cells (GFAP antigen [E]). Arrows indicate a GFAP-positive astrocyte (E) which is MHV3 negative (D). Magnification, ×160).

ble C57/BL6 mice (brain extracts were prepared as for viral titration and diluted 1:10 in PBS solution, and 0.1 ml of these suspensions was injected i.p.). The measured viral titers in the liver increased during the first 3 days p.i. up to a titer of 2×10^4 PFU/g. After day 7 p.i., no viral activity could be detected either by plaque assay or injection into susceptible mice.

Viral titers and the rate of viral replication in the livers of similarly infected A/Jx mice (which are apparently resistent to MHV3-induced disease) were not significantly different from those observed in C3H mice. Marked differences, however, were observed for viral penetration in the brains of A/Jx compared with that of C3H mice. Thus, A/Jx mice injected with 10^2 PFU had less than 5×10^2 PFU of virus per g of brain. Virus was detected in the brain of A/Jx mice on day 4 p.i. only after injection of 10^4 PFU of virus (Table 2).

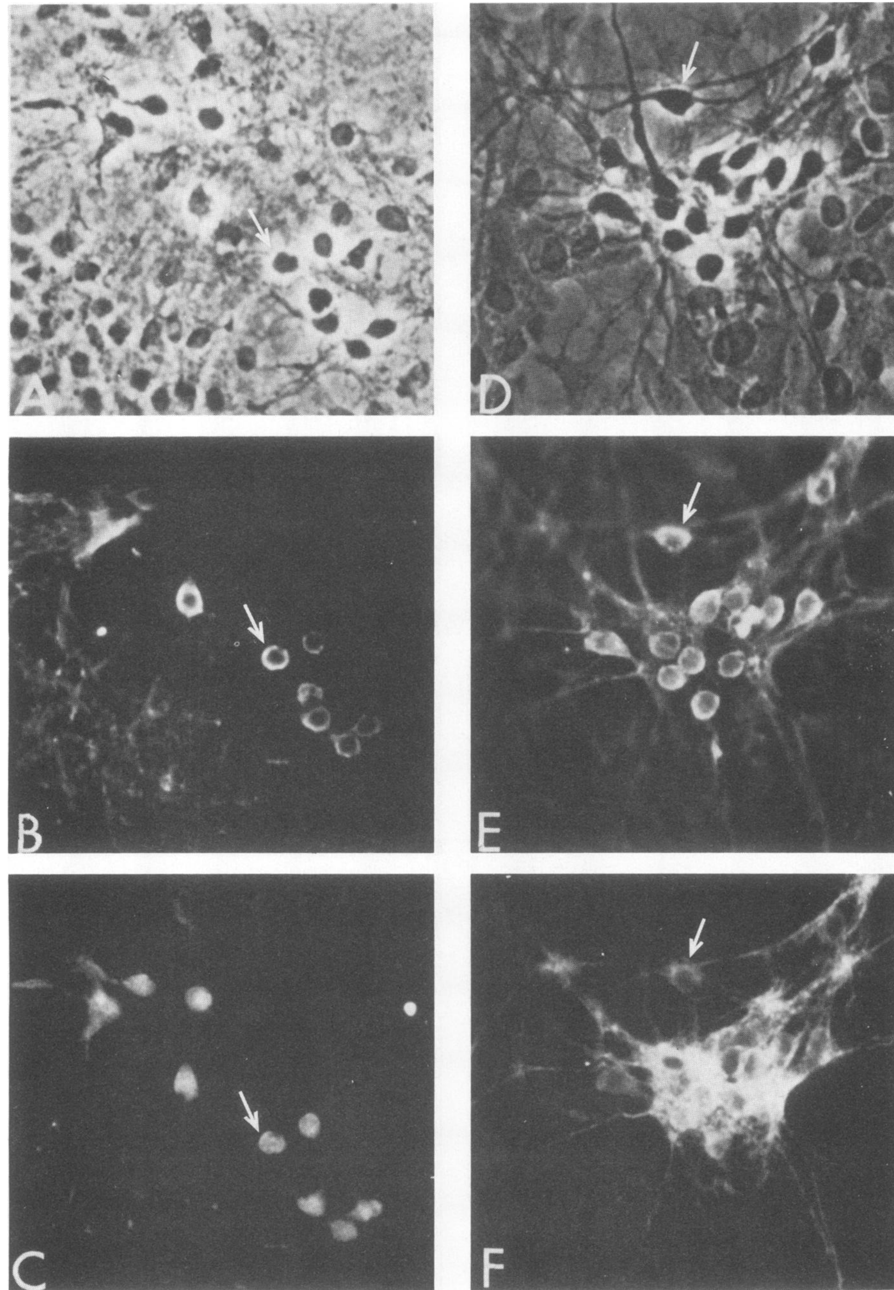


FIG. 4. Immunofluorescent localization of MHV3, A2B5, and N-Cam antigens in MHV3-infected fetal cortical cell cultures. Cortical cell cultures were tested 48 h p.i., fixed, and treated with rabbit anti-MHV3 antibody and rhodamin-conjugated Garig, followed by mouse anti-A2B5 antibody (or anti-N-Cam antibody) and FITC-Gamig. In cultures as seen under a phase-contrast microscope (A and D), rhodamin-positive cells (MHV3 antigen [B and E]) were also fluorescence positive (A2B5 antigen [C] or N-Cam antigen [F]). Arrows indicate examples of two neurons that were MHV3 positive (B and E) and either A2B5 positive (C) or N-Cam positive (F). Magnification, ×230).

No neuropathological lesions were observed in brains of A/Jx mice infected with 10^4 PFU of MHV3 (data not shown), despite virus titers in brains that were as high in this group as in C3H mice with CNS infections. No persistent virus was detectable in the organs of infected A/Jx mice after day 7 p.i.

Viral titers in the livers of infected C57/BL6 mice (which develop a lethal acute hepatitis) were significantly higher ($P < 0.005$) than in the livers of either C3H or A/Jx mice, whereas viral titers in brains were similar to titers obtained in C57/BL6 and C3H mice.

In a subsequent series of experiments, 10^2 PFU of virus were injected directly into cerebral tissue of C3H, A/Jx, or C57/BL6 mice. Viral titers were identical in the brains of all mouse strains on day 4 p.i.: $4.2 \times 10^4 \pm 2.4$ PFU/g (A/Jx), $4.2 \times 10^4 \pm 4.1$ PFU/g (C3H), and $4.2 \times 10^3 \pm 1.4$ PFU/g (C57/BL6).

Affinity of purified MHV3 for isolated ependymal cells and oligodendrocytes. (i) C3H mice. Binding of purified MHV3 was first tested on L cells which are known to replicate the virus. A viral titer of at least 5×10^6 PFU/ml was required to stain more than 75% of the tested cells. Accordingly, this titer was used in all the subsequent experiments.

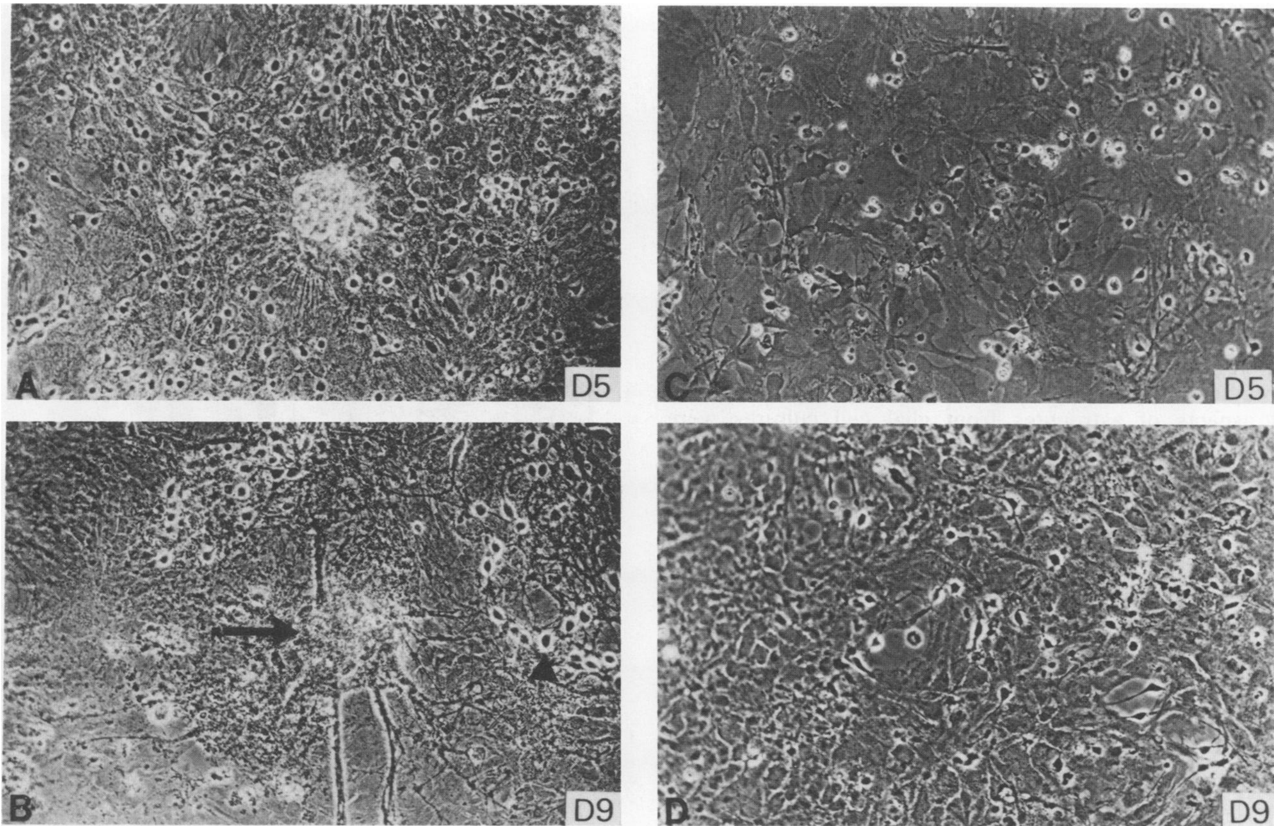


FIG. 5. Maturation of cortical neuronal cells or cortical glial cells in cultures with MHV3. Cortical cells in a culture obtained from 15-day-old embryos were infected 11 days postplating with 10^2 PFU per well of MHV3 and are shown at 5 and 9 days p.i. Cultures were unaffected by MHV3 infection at 5 days p.i. (A). At 9 days p.i., some neurons were disrupted (arrow), whereas some others were still morphologically unaltered (arrowhead) (B). Cortical glial cells in a culture obtained from 1-day-old neonates were similarly infected 6 days postplating. Cultures are shown at 5 (C) and 9 (D) days p.i. and appear identical. Glial cells were morphologically unaltered after MHV3 infection. Magnification, ×25).

Purified MHV3 bound to 82% of isolated ciliated ependymal cells but only to 25% of cells present in the oligodendrocyte-enriched cell suspension, as judged by indirect immunofluorescence (Table 3 and Fig. 1). In the oligodendrocyte-enriched cell suspension, 51% of the cells were labeled by an anti-galactocerebroside antibody and were presumably oligodendrocytes, 39.5% of the cells were GFAP positive, and 10.5% were recognized by tetanus toxoid. As judged by double labeling with anti-galactocerebroside and anti-MHV3 antibodies, 23% of the anti-galactocerebroside-positive cells were double labeled by anti-MHV3 antibodies.

Viral binding on ependymal cells was predominantly observed at the bottom of cilia and on the cilia themselves (Fig. 1), and the fluorescence pattern was typical of membrane staining. The difference observed between ependymal cells and oligodendrocytes was even more striking when ependymal cells were occasionally observed in the oligodendrocyte cell suspension: the ciliated ependymal cells were strongly fluorescent, whereas round oligodendrocytes cells were not.

(ii) Sensitive versus resistant strains. Ependymal cells isolated from the brains of C3H, A/Jx, or C57/BL6 mice were compared for their abilities to bind purified MHV3. The percentages of labeled cells were identical when ependymal cells from resistant or susceptible mice were tested. Thus, 80% of the isolated ependymal cells from C3H mice were labeled, as compared with 83% of the cells from either A/Jx or C57/BL6 mice.

Affinity of purified MHV3 for cultured cortical neurons and astrocytes. (i) Virus binding and penetration. Fetal cortical neurons were brightly stained by indirect immunofluorescence with purified MHV3 and the corresponding antibodies (Fig. 2). Viral binding was observed on the surface of the majority of examined neurons, on cell bodies, and on neurites. Neurons of different ages (3 to 20 days in cultures) and of different genetic background (C3H or A/Jx) were equally stained. In sharp contrast, the nonneuronal cells (the vast majority of which were GFAP positive) were not stained during the procedure (Fig. 2).

When similar cultures were infected with 10^2 PFU of MHV3 and were tested 24 or 48 h later for the presence of intracytoplasmic MHV3 antigens, 80 to 85% of the neurons in five different experiments were stained, whereas nonneuronal cells were negative (Fig. 3A and B). A few neuronal cells appeared to be still negative after 48 h in virus-infected cultures. Moreover, MHV3-infected glial cell cultures obtained from newborn mice were negative for the presence of intracytoplasmic viral antigen, except for a small percentage of cells (4 to 6%) in cultures tested more than 4 days p.i. (in eight different experiments, with cultures tested from days 1 to 6 p.i.).

Cultured cells were subsequently double labeled for the presence of intracytoplasmic MHV3 antigen and GFAP,

A2B5, or N-Cam antigen. No GFAP-positive cell was found to be positive for MHV3 antigen (Fig. 3C, D, and E). On the other hand, more than 80% of the A2B5-and N-Cam-positive cells were MHV3 antigen positive (Fig. 4).

(ii) Selective effect of MHV3 on neurons and astrocytes. The morphological maturation of cells in virus-infected and noninfected cortical neuronal cell cultures was similar for 5 to 6 days p.i., after which neurons appeared progressively disrupted over a 3-day period but astrocytes continued to proliferate. When a fresh suspension of fetal cortical cells was incubated for 60 min with 10^2 PFU of virus, similar results were observed: neurons matured normally during the first 6 days p.i., after which their cell bodies and neurites degenerated, whereas the morphological aspect of astrocytes was preserved (Fig. 5). Similarly, the morphological aspects of astrocytes from glial cell cultures were identical in infected (10^2 PFU per culture) and uninfected cultures (Fig. 5). Some astrocyte disruptions without fusions were, however, observed when cultures were infected with more than 10^4 PFU of virus per culture (multiplicity of infection > 0.01).

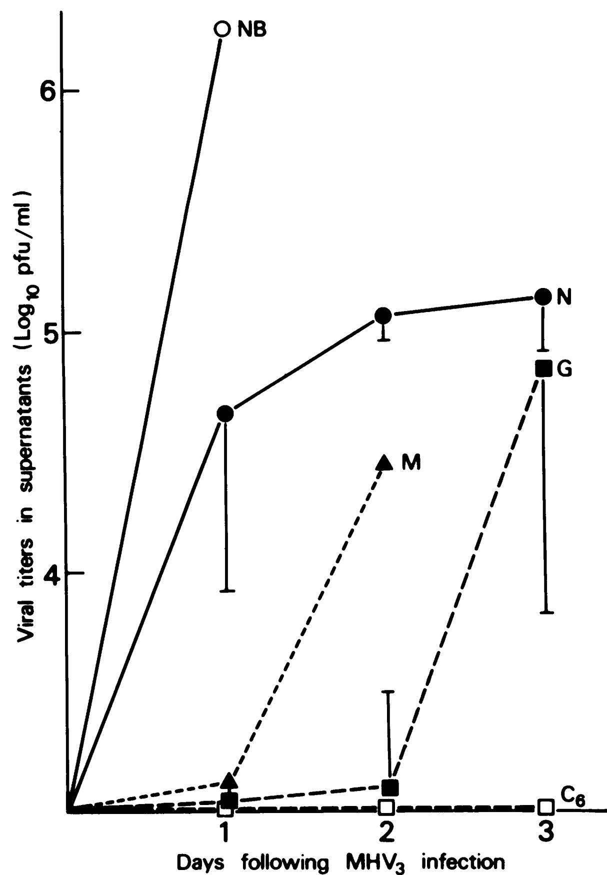
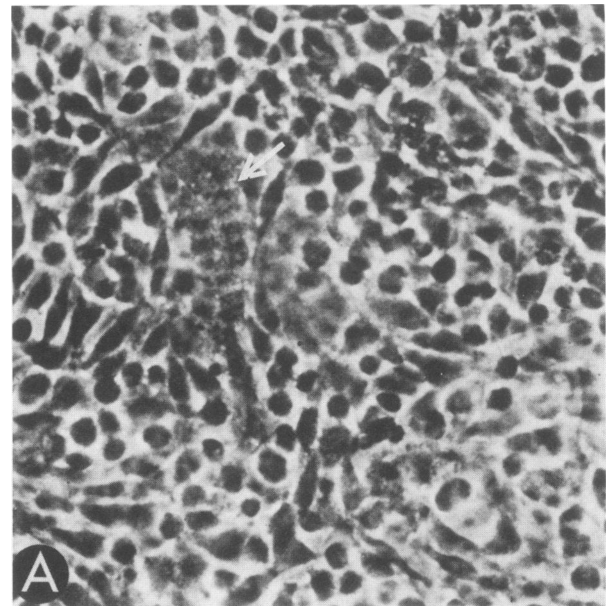


FIG. 6. Viral titers in supernatant of cultured cells during the first 3 days p.i. ○, Supernatants obtained from neuroblastoma (NB) cells (NIE 115 cell line) (cellular monolayer was destroyed between days 1 and 2 p.i.); ●, supernatants obtained from young fetal cortical cell cultures containing mainly neurons (N) (each point represents the mean ± 1 standard deviation of 10 experiments); □, supernatants obtained from C6 glioma cells (C_6); ■, supernatants obtained from neonatal cortical cell cultures containing mainly glial cells (G) (each point represents the mean ± 1 standard deviation of 11 experiments); ▲, supernatants obtained from meningeal cells (M) (cellular monolayer was destroyed after day 2 p.i.). All cultures were infected with 10^2 PFU of MHV3 per 10^6 cells.



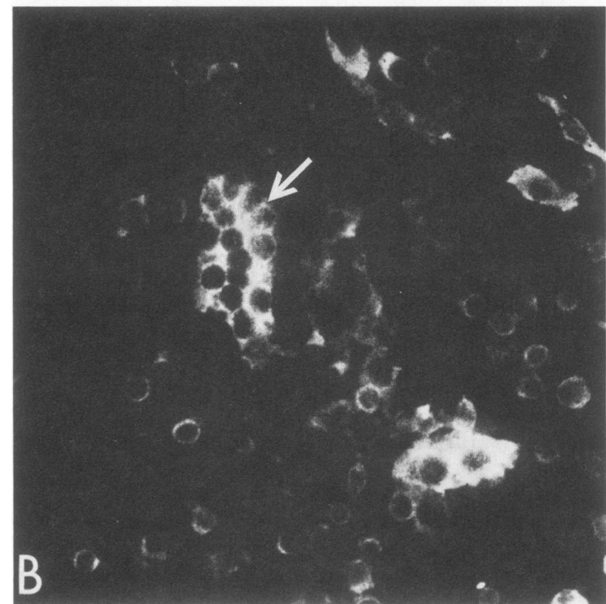


FIG. 7. Immunofluorescent localization of MHV3 antigens in infected meningeal cells. Meningeal cells were tested at transfer 3. Cells were infected with 10^2 PFU of MHV3 and are shown 24 h after infection. Large syncytia, which can be observed with a phase microscope (A), contain MHV3 antigen as indicated by indirect immunofluorescence (B). Magnification, ×83).

To further analyze the respective susceptibilities of astrocytes and neurons to MHV3, we tested two continuous cell lines, one of glial origin (C6 glioma) and the other of neuronal lineage (neuroblastoma NIE 115). Neuroblastoma cells, when infected at a multiplicity of infection of 10^{-4}, were destroyed within 36 h without syncytia formation. In sharp contrast, C6 glial cells appeared unaffected by MHV3 infection.

Viral titers in supernatants of 3- to 6-day-old neuronal cultures or of neuroblastoma cell cultures increased sharply during the first days of infection. Conversely, no virus was

found in the supernatant of infected C6 glioma cells, whereas viral titers increased slowly in supernatants of glial cell cultures (Fig. 6). At days 1 and 2 p.i., viral titers in the supernatants of infected glial cells were significantly lower than those observed in the supernatants of infected neuronal cultures. By day 3 p.i., viral titers in supernatants of both types of cultures were identical.

Susceptibility of meningeal cells to MHV3. Meningeal cells in cultures appeared very susceptible to MHV3. When cultures were infected with 10^2 PFU of MHV3, many meningeal cells formed large syncytia after 12 h and were disrupted within 48 h. Intracytoplasmic viral antigen was detected by indirect immunofluorescence in the cytoplasm of all the syncytial formations (Fig. 7). Viral titers in the supernatant increased to 2.8×10^4 PFU/ml (Fig. 6).

DISCUSSION

Interaction of viruses with target cells requires that the virus first reach and bind to the cells; this initiates the chain of events leading to viral infection. The present series of experiments investigated the ability of a neurotropic virus, MHV3, to invade the CNS and to recognize cells selectively within the brain. We observed that purified MHV3 has an in vitro affinity for neurons, ependymal cells, and meningeal cells but not for astrocytes and oligodendrocytes. This corresponds to its pattern of pathogenicity in vivo, because MHV3 induces an initial ependymitis, meningitis, and encephalitis in the absence of the white matter lesions (21, 23). The difference between neurons and astrocytes in susceptibility to MHV3 was striking. No viral surface binding was observed on astrocytes by indirect immunofluorescence, whereas neurons in the same culture were brightly stained. Viral antigens were observed only in neuronal cells at 24 h p.i. Neurons but not astrocytes appeared to be slowly destroyed after viral infection, and viral replication was very active in the supernatant of young embryonic cultures when neuronal cells predominated. Moreover, C6 glioma cells were morphologically resistant to and did not replicate MHV3, whereas neuroblastoma cells were highly susceptible to MHV3. Glial cells in cultures, however, retained some susceptibility to MHV3 since evidence of slow viral replication was observed and cellular change could be produced if the cultures were infected with a viral inoculum of high titer.

MHV4 (JHM strain), another member of the mouse hepatitis virus group, differs from MHV3 in its affinity for CNS cells in vivo. MHV4 induces encephalitis and under certain conditions (for example with a temperature-sensitive mutant), a demyelinating disease (9). A good correlation was found in vitro, because JHM wild-type virus, which induces encephalitis, infected both neuronal and nonneuronal cells in mouse spinal cord cultures, whereas the temperature-sensitive mutant infected only nonneuronal cells (9, 11). It is interesting that both wild-type JHM and the temperature-sensitive mutant have a strong affinity for astrocytes, whereas MHV3 has a low affinity for them. Similarly, strain A59 (which induces in vivo hepatitis and demyelination) lacks tropism for neurons and primarily infects nonneuronal cells in vitro, the reverse pattern of that observed with MHV3 (3, 6, 12). In our own system with cortical cell cultures, we repeated the same experiment with strain A59 (a kind gift from K. Holmes) and confirmed the binding of A59 virus to glial cells. Moreover, A59 seems to bind to ciliated ependymal cells, as well as to galactocerebroside-positive oligodendrocytes (unpublished results). Restricted tropism of wild-type JHM, its temperature-sensitive mutant, strain A59, and MHV3 for CNS cells could be due to a change in the viral glycoprotein responsible for cellular attachment (polypeptide E2). Recent studies with monoclonal antibodies have demonstrated that polypeptide E2 has no major antigenic conservation among the wide range of murine coronaviruses, even if other MHV3 and A59 polypeptides appear antigenically very close (7).

Correlations between the in vivo pathogenicity of a virus and its in vitro affinity for isolated or cultured CNS cells have been established in different models of viral CNS infection (3, 5, 6, 20). Thus, reovirus type 1, which induces ependymitis in vivo, binds to murine and human ependymal cells, whereas reovirus type 3, which induces encephalitis, does not (22). Conversely, a subpopulation of neurons was shown to bind reovirus type 3, as opposed to reovirus type 1 (4). However, both serotypes bind to and infect astrocytes. Cultures infected by both serotypes show a cytopathic effect by 24 h and are disrupted by day 5 p.i. (4). The specificities of reovirus type 3 for neurons and of reovirus type 1 for ependymal cells were a property of the viral hemagglutinin, as in vivo (4, 22, 24).

Neurologic infection induced by coronaviruses, including MVH3, is under genetic control. A/Jx mice are resistant to acute and chronic diseases induced by MVH3, whereas C3H mice express neurologic diseases and neuropathological lesions. We observed that MHV3 invaded the CNS of both strains of mice, although in A/Jx mice, higher titers of virus were needed to overcome peripheral resistance. Nevertheless, in this strain of mice, no neuropathological lesions developed in spite of CNS viral penetration. In vitro, viral binding of susceptible and resistant strains to adult ependymal cells was identical, suggesting that the resistance of ependymal cells to viral infection was not due to the absence of a specific receptor for virus in the resistant strain, a result in accordance with a previous report of Knobler et al. (11) on genetic control of neuronal resistance to MHV4 and of Arnheiter et al. (1) on hepatocyte resistance to MHV3.

The specific affinity of MHV3 for ependymal cells and neurons permits an in vitro approach to virus-CNS cell interaction. Of particular interest is the selective infection of cultured neurons with the preservation of astrocytes, since a destruction of astrocytes induces by itself a disruption of neuronal cultures. This, associated with the delayed expression of virus-induced neuronal lesions, provides a way to study metabolic activities and, more specifically, neuromodulating activities of virus-infected neurons (2).

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