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The V5A13.1 Envelope Glycoprotein Deletion Mutant of Mouse Hepatitis Virus Type-4 Is Neuroattenuated by Its Reduced Rate of Spread in the Central Nervous System

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Following intracerebral inoculation of adult Balb/c Byj mice, the MHV-4 strain of mouse hepatitis virus (MHV) had an LD₅₀ of <0.1 PFU, whereas its monoclonal antibody resistant variant V5A13.1 had an LD₅₀ of 10^{4.2} PFU. To determine the basis for this difference in neurovirulence we have studied the acute central nervous system (CNS) infection of these two viruses by *in situ* hybridization. Both viruses infected the same, specific neuroanatomical areas, predominantly neurons, and spread via the cerebrospinal fluid, along neuronal pathways and between adjacent cells. The neuronal nuclei infected and the spread of virus within the brain are described. The main difference between the parental and variant viruses was the rate at which the infection spread. MHV-4 spread rapidly, destroying large numbers of neurons and the animals died within 4 days of infection. The variant virus spread to the same areas of the brain but at a slower rate. This difference in the rate of virus spread was also apparent from the brain virus titers. The slower rate of spread of the variant virus appears to allow intervention by the immune response. Consistent with this, the variant virus spread slowly in athymic *nu/nu* mice, but in the absence of an intact immune response, infection and destruction of neurons eventually reached the same extent as that of the parental virus and the mice died within 6 days of infection. We conclude that the V5A13.1 variant of MHV-4 is neuroattenuated by its slower rate of spread in the CNS. © 1992 Academic Press, Inc.

INTRODUCTION

Mouse hepatitis virus (MHV) is presently studied as a model of both acute and chronic central nervous system (CNS) disease where the outcome of infection can range from acute encephalitis to chronic demyelinating disease (for reviews see Siddell *et al.*, 1983; Wege *et al.*, 1986; Buchmeier *et al.*, 1987).

Recent sequence data on the S gene of the JHM, A59, and MHV-4 strains (Schmidt *et al.*, 1987; Luytjes *et al.*, 1987; Parker *et al.*, 1989) have rendered the interpretation of the pathogenesis of MHV considerably more complex. It is now clear that the genome of this virus is highly variable, in particular, an area of hypervariability is present in the S1 gene (Parker *et al.*, 1989). This hypervariable region undergoes high frequency recombination (Banner *et al.*, 1990) and both deletions and recombinations may result from "polymerase jumping" (Lai *et al.*, 1987; Lai, 1990). The consequence of this hypervariability is that MHV propagated and studied in one laboratory may be very different from that in another. Furthermore, if the virus is regularly passaged it may vary over time and variant viruses have been readily obtained by passage of virus

stocks *in vivo* and *in vitro* (Taguchi *et al.*, 1985, 1986; Morris *et al.*, 1989; Gallagher *et al.*, 1990).

As well as the strain of the virus, the outcome of virus infection of the CNS is dependent on the route and dose of infection and the age, species, and strain of the rodent. This can be seen by comparison of the neurovirulence of the three strains of MHV for which the S gene has been sequenced. Four-week-old Balb/c mice are highly susceptible to MHV-4 or MHV-JHM; the intracerebral (ic) LD₅₀'s are <0.5 PFU and <0.1 PFU, respectively (Knobler *et al.*, 1981a; Wege *et al.*, 1988). SJL/J mice are less susceptible to MHV-4 (Dalziel *et al.*, 1986) and even less susceptible to MHV JHM (Stohlman and Frelinger, 1978). MHV A59 virus is less neurovirulent than MHV-4 or MHV JHM (Robb *et al.*, 1979; Lavi *et al.*, 1986).

In contrast to the acute encephalitis, a subacute or chronic demyelinating encephalitis develops in occasional mice surviving ic infection with MHV-4, MHV JHM, or a high dose of MHV A59 (Lampert *et al.*, 1973; Knobler *et al.*, 1982; Erlich and Fleming, 1985; Lavi *et al.*, 1986) and in animals protected from acute infection by neutralizing antibodies (Buchmeier *et al.*, 1984; Perlman *et al.*, 1987). The course of CNS disease has also been studied for several viruses derived in the laboratory by tissue culture passage, by selection for temperature sensitivity, or by selection for antibody resistance. These laboratory variants include MHV-4, ts8 (Haspel *et al.*, 1978); MHV-4, V5A13.1 (Dalziel *et al.*,

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1986); MHV-A59, ts342 (Koolen *et al.*, 1987); MHV-JHM, Pi (Baybutt *et al.*, 1984); MHV-JHM, CC (Hirano *et al.*, 1981); MHV-JHM, ts43 and B35 (Wege *et al.*, 1983, 1988); and MHV-DL, 2.2-V-1 and 2.2/7.2-V-2 (Fleming *et al.*, 1987). When inoculated intracerebrally into susceptible strains of mice, relative to their parental viruses, all of these variants are neuroattenuated and produce a nonlethal demyelinating encephalitis.

The S envelope glycoprotein of MHV-4 is responsible for attachment to cells and for viral fusion and is also the main target of neutralizing antibodies (Collins *et al.*, 1982; Sturman *et al.*, 1985). Relative to the S protein of MHV-4, MHV-JHM and MHV-A59 have deletions of 141 and 52 amino acids, respectively, in the S1 protein (Schmidt *et al.*, 1987; Luytjes *et al.*, 1987; Parker *et al.*, 1989). In the case of the various neuroattenuated variants of MHV, the only sequence data available are for the S gene of the antibody-resistant variants of MHV-4 (Parker *et al.*, 1989). These variants were selected for their ability to resist neutralization by monoclonal antibodies directed against the MHV-4 S protein (Dalziel *et al.*, 1986). The S1 glycoprotein of the V5A13.1 variant differs from that of MHV-4 wild-type (wt) by the deletion of 142 amino acids, residues 434–575 of the wt protein (Parker *et al.*, 1989; Gallagher *et al.*, 1990).

In the present study, we focus on understanding the basis for the difference in neurovirulence between our strain of MHV-4 and its V5A13.1 variant. This is presently the only system where the genotype of a neurovirulent strain of MHV and its neuroattenuated variant are both defined at the S locus. For this pair of viruses, we have determined the infected neuronal nuclei, the pathology, the likely mechanism by which the viruses spread within the CNS, and the basis for the differential outcome of these infections.

MATERIALS AND METHODS

Virus

MHV-4 (wt) was originally obtained from L. P. Weiner (University of Southern California, Los Angeles, CA) and has been maintained in this laboratory by several passages in SAC⁻ cells.

The isolation and characterization of the V5A13.1 variant from MHV-4 (wt) has been described previously (Dalziel *et al.*, 1986). The variant used in the experiments described here is the original V5A13.1 (86) antibody-resistant variant derived in 1986. V5A13.1 (86) has been maintained in the laboratory by passage in SAC⁻ cells. The stock of virus used in the present study is one passage in SAC⁻ cells from a stock used for RNA sequencing of the S gene (Parker *et al.*, 1989). Sequencing of this new stock detected no changes from the published sequence.

Infection of mice

BALB/c Byj and BALB/Wehi *nu/nu* mice from the Scripps Clinic breeding facility were infected intracerebrally with virus at 6 to 8 weeks of age. Animals were anesthetized and virus inoculated intracerebrally, 100 PFU in 0.05 ml of sterile saline. Brains were removed at various times postinfection and divided sagittally. One-half was frozen for virus titration. The other half of the brain was placed in 10% phosphate-buffered formalin and after 1 to 2 days processed by routine techniques and embedded in paraffin. Sagittal sections 5- μ m thick were cut and mounted in triplicate on poly-L-lysine coated slides.

Three brains on each of Days 1 to 4 and at least six sections from each were processed for *in situ* hybridization.

Virus titers

Samples for virus titration were stored at -70° until assayed. Tissues were homogenized (10% w/v) in Dulbecco's minimal essential medium with 7.5% heat-inactivated fetal calf serum. Infectivity was determined by plaque titration on DBT cells (Hirano *et al.*, 1978).

In situ hybridization

The technique was based on that described by Reynolds-Kohler and Nelson (1990). Briefly, paraffin sections were dewaxed for 15 min in Americlear (American Scientific Products); hydrated through graded alcohols containing 0.33 M NH₄ acetate; washed in water; and treated sequentially with 0.2 M HCl (10 min), 1% Triton X-100 (1.5 min), 10 μ g/ml proteinase K in 20 mM Tris, 2 mM CaCl₂, pH 7.0, buffer (20 min, 37 $^{\circ}$), and then 0.25% acetic anhydride in 0.1 M triethanolamine, pH 8.0 (10 min). Finally, sections were dehydrated through alcohols containing 0.33 M NH₄ acetate and left to air dry. All treatments were at room temperature unless indicated. Sections were washed between each treatment (2 \times 3 min) with sterile phosphate-buffered saline (PBS). The PBS wash after proteinase K digestion contained in addition 0.2% glycine and 5 mM EDTA to stop the reaction. Before use, glassware and slide racks were thoroughly cleaned in a solution of 0.2% SDS, 0.2% EDTA and finally washed in sterile water. The hybridization solution was 50% deionized formamide, 5 \times Denhardt's solution, 10% dextran sulfate, 0.1% SDS, 0.75 M NaCl, 0.025 M Pipes, 0.025 M EDTA, 500 μ g/ml sonicated salmon sperm DNA, 250 μ g/ml yeast tRNA (boiled before addition), 20 U/ml heparin and was freshly made each time. Sections were prehybridized for 1 hr at 37 $^{\circ}$ with 150 μ l of the above solution without dextran sulfate.

Slides were drained and 25 μ l of hybridization solu-

tion containing 2×10^5 cpm/ μ l of probe was added to each. The sections were covered with gel bond (FMC BioProducts, Rockland, ME), hydrophobic side down, and sealed with rubber cement. Hybridization was at 55° overnight. These conditions resulted in strong signal with minimal background.

Cover slips were removed in 4× SSC (1× SSC is 0.15 M NaCl, 0.015 M Na citrate) containing 50 μ M DTT. The slides were washed in RNAase digestion buffer (2 × 10 min, 37°, 0.5 M NaCl, 10 mM Tris–Cl, 1 mM EDTA, pH 8.0). Digestion (30 min, 37°) was carried out in the above buffer by the addition of 50 μ g/ml RNase A. Sections were then washed in fresh RNase digestion buffer (4 × 15 min, 37°) and finally washed in 0.1× SSC (2 × 15 min, 55°). Sections were air dried and exposed to high resolution Cronex film (Dupont, Wilmington, DE) before dipping in Kodak NTB2 emulsion, diluted 50% with 0.66 M NH₄ acetate. Exposure was for 4 to 7 days at 4°. Finally, sections were counterstained with hematoxylin and eosin and examined by bright and dark field microscopy.

RNA probes labeled with [³⁵S]ATP and -CTP) were used in all experiments described here. A recombinant plasmid containing a 1.8-kb cDNA fragment from the 3' end of the MHV A59 genome, representing genes 5 and 6 and 200 nucleotides of genes 4 and 7, cloned into pGem 3, was kindly provided by Dr. Susan Weiss (Department Microbiology, University of Pennsylvania). The plasmid was linearized at the *Hind*III site of the vector and anti-sense RNA transcripts produced by *in vitro* transcription from the T7 promoter (using a Promega riboprobe Gemini kit, Promega Corp., Madison, WI). The RNA was hydrolyzed to a size range of 200 bases by incubation, 30 min at 60° in 0.04 M NaHCO₃. The hydrolysis was stopped by neutralization with 1/10 vol of 3 M Na acetate, pH 4.6. Probes were ethanol precipitated, resuspended in diethylpyrocarbonate-treated water, and stored at –20° until use. Probes routinely had a specific activity >10⁸ cpm/ μ g. For use, probes were boiled for 3 min, DTT was added to 10 mM, and the probe was added to the hybridization mix to give 2×10^5 cpm/ μ l.

RESULTS

LD₅₀ and brain virus titers

We determined the intracerebral LD₅₀ of these viruses in 6- to 8-week-old BALB/c Byj mice to be <0.1 PFU (0.05 ml) for MHV-4 and 1.6×10^4 PFU for V5A13.1.

The titers of virus in the brain are shown in Table 1 and indicate a clear difference between the two viruses. As early as 1 day postinfection, the titer of wt virus was 3.2×10^4 PFU/g, but the variant virus could not be detected until 2 days after infection. Viral titers

TABLE 1
COMPARISON OF TITERS OF MHV-4 (wt) AND V5A13.1
DELETION MUTANT IN THE BRAINS OF BALB/C MICE

Days postinfection ^a	Brain virus titer (PFU × 10 ³ /g) ^b	
	MHV-4 (wt)	V5A13.1
1	35	<0.4
2	2000	49
3	1400	100
4	NA ^c	10
7	NA	<0.4

^a Six- to 8-week-old adult Balb/c Byj mice were infected intracerebrally with 100 PFU of either MHV-4 (wt) or V5A13.1 virus in 0.05 ml sterile saline and sacrificed for virus titration at the intervals indicated.

^b Virus titers are given as PFU/g brain tissue and represent the mean of three mice. The limit of detection in this assay was 400 PFU/g brain tissue.

^c NA, no mice survived for virus titration at these times.

in the brains of V5A13.1-infected animals remained well below those of wt, 40-fold less at 2 days and 14-fold less at 3 days. By the fourth day, the V5A13.1 titers in the brain had dropped dramatically. Variant virus could not be detected by plaque assay at 7 days.

In situ hybridization

The specificity of the MHV probe was tested on sections of uninfected brain tissue and sections from the brains of mice persistently infected with lymphocytic choriomeningitis virus (LCMV), both processed exactly as for the MHV-infected brains. The MHV probe did not hybridize to these control tissues nor did a probe to the LCMV nucleoprotein gene hybridize to MHV-infected brain (data not shown).

Representative sections showing the distribution of viral nucleic acid on Days 1 to 4 postinfection in the brains of mice infected intracerebrally with MHV-4 (wt) and V5A13.1 are shown in Fig. 1. It is evident from these sections that wt virus spread rapidly producing extensive involvement of large areas of the cortex and brain stem. In contrast, although the V5A13.1 virus infected the same areas of the brain it did so at a slower rate, with less extensive involvement of these areas. This slower rate of spread of the variant is consistent with the lower virus titers in the brain (Table 1).

Six sagittal sections from each of three mice on Days 1 to 3 and 20 sections from each of two mice on Day 4 were examined. The following describes the consistently observed features of the extent and distribution of the MHV-4 (wt) and V5A13.1 infections from Days 1 to 4. There was little variability between animals, and this was more in the extent to which a particular nu-

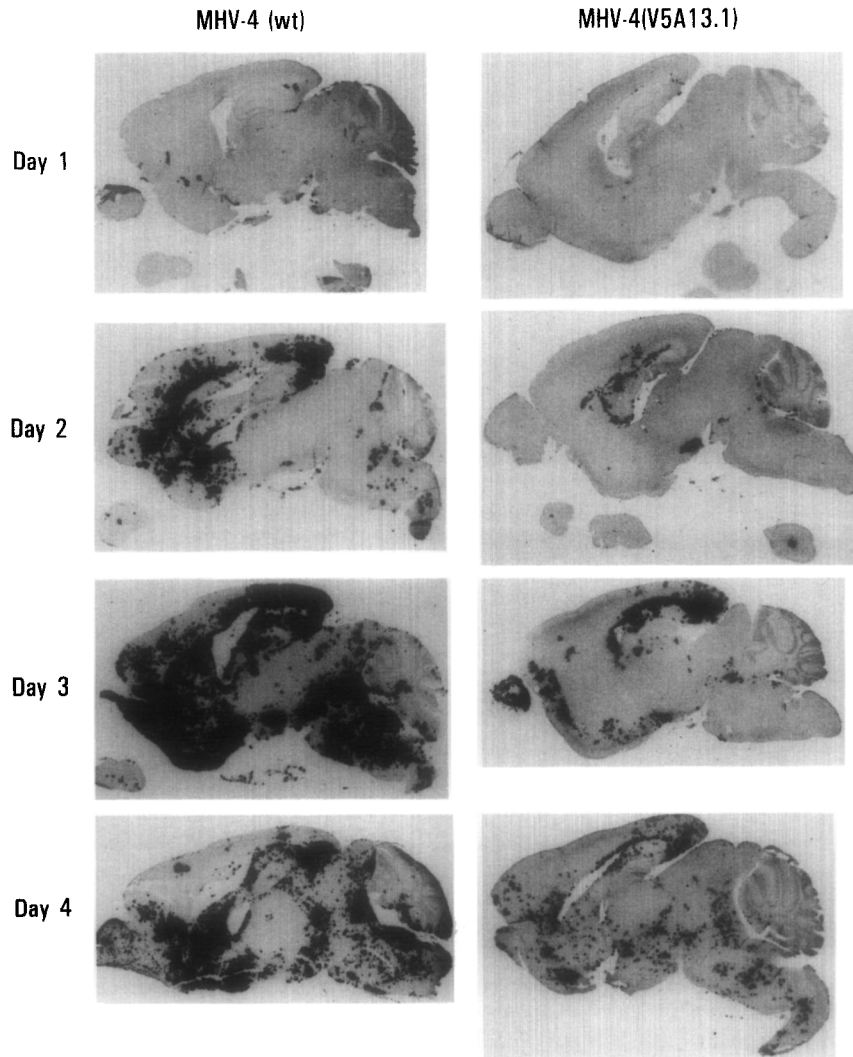


FIG. 1. Distribution of viral nucleic acid detected by *in situ* hybridization in sagittal sections from the brains of mice infected intracerebrally with 100 PFU of MHV-4 (wt) or its V5A13.1 variant. Transverse sections of spinal cord are also present showing infection around the spinal canal. Tissues are 5- μ m paraffin sections and images were photographed from cronex film. Most mice infected with MHV-4 (wt) were dead by Day 4. The brain shown on Day 4 is from one of two mice that survived until this time.

cleus was infected at a particular time point than in the nuclei infected. These differences within each virus group were minor compared to those observed between the different viruses.

MHV-4 (wt) infection

Signal was first observed at Day 1 postinfection in a few cells of the deep cortical layers near to the site of infection; these were predominantly pyramidal cells. By 2 days postinfection the signal in the deep layers of the striatum cortex had spread posteriorly to involve many cells in the retrosplenic cortex and the subiculum and anteriorly via the dorsal transition zone and the anterior olfactory area into the olfactory lobe. In the olfactory lobe periglomerular cells and cells in the mitral cell layer were infected (Figs. 2C and 2D). Nuclei in

the rhinencephalon and cells in the olfactory tubercle connecting these to the olfactory lobe were also positive.

Areas known to receive projections from the positive deep cortical areas were positive. These included the caudate, the basal ganglia, and the anterior area of the pons. In some animals a few positive cells were present in these areas as early as Day 1. Other areas variously infected in different mice included foci in the inferior colliculus, the superior olive, the nucleus accumbens, the medial vestibular, cuneiform, intrape-duncular and arcuate nuclei, and the mammillary bodies. By 2 days postinfection two of the three animals showed infection of neurons in the CA1 and CA3 layers of the hippocampus (Fig. 3A) and in one animal signal was evident in the molecular layer. In all animals, the

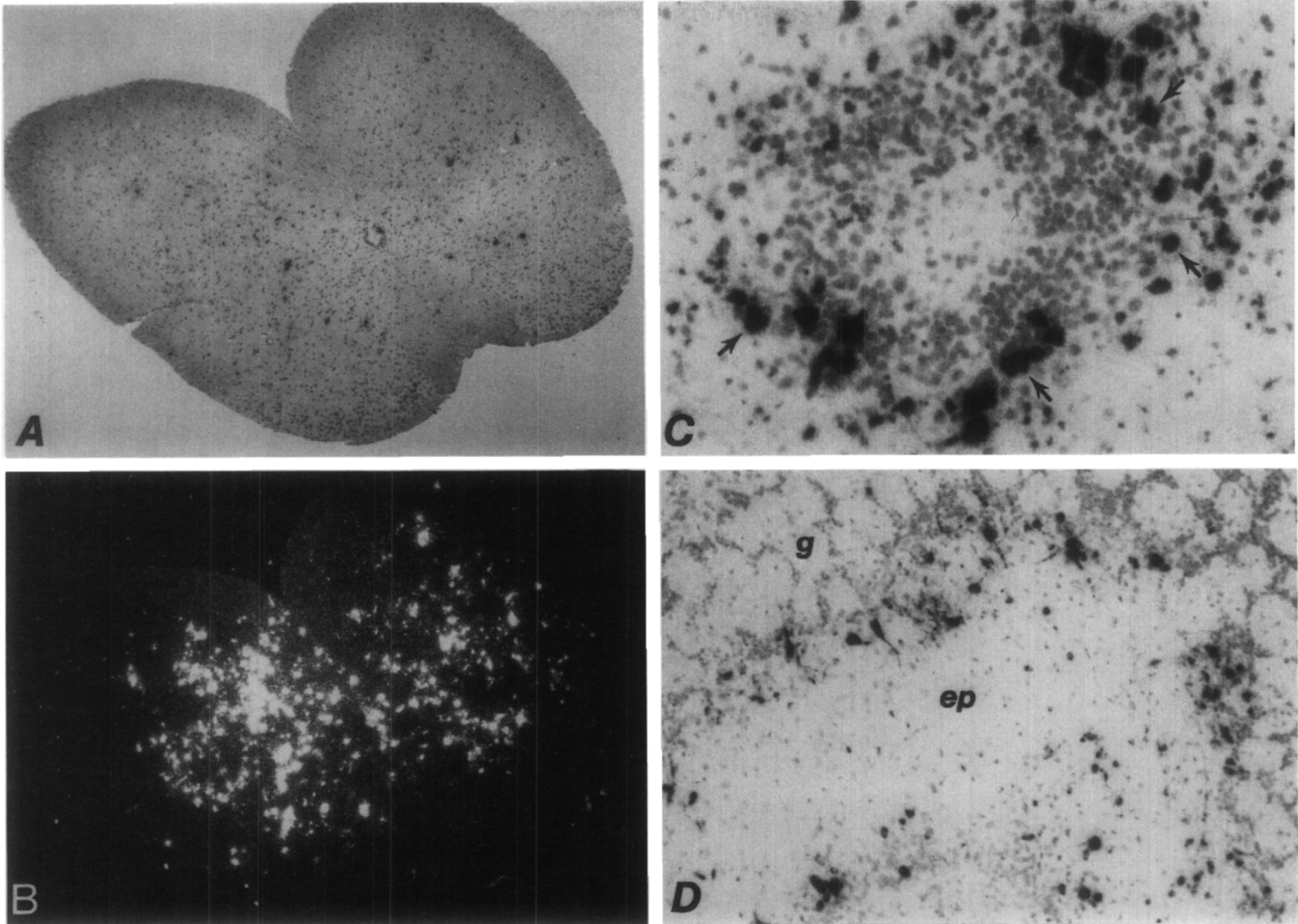


FIG. 2. Microscopic localization of viral nucleic acid in the spinal cord and olfactory bulb of mice infected intracerebrally with MHV-4. Sections were analyzed by *in situ* hybridization and photographic emulsion autoradiography as described under Materials and Methods. (A and B) Light and dark field microscopy of the same coronal section of spinal cord; 3 days postinfection. (C) Infection of mitral cells (arrowed) in the olfactory bulb; 2 days postinfection. (D) Infection of cells at the junction of the exterior plexiform (ep) and glomerular cell layers (g), 2 days postinfection.

dentate gyrus remained relatively uninvolved with only occasional positive cells. Infection of a nucleus in the hypothalamus, probably the supramammillary or subthalamic nucleus, was one of the most consistent features of infection and was apparent in all mice by Day 2 (Fig. 4B). Foci of infection were present in the brain stem and pons. In some animals the cerebellar peduncle, the deep cerebellar nucleus, and occasional Purkinje cells were positive.

In the spinal cord, signal was apparent in neurons adjacent to the spinal canal (Fig. 1). Infection of ependymal cells was rare. Occasionally groups of three or four positive, adjacent ependymal cells were observed lining the third or lateral ventricles. In some cases the infection appeared to have spread to the underlying neurons. Certain areas lacked signal, including the outer layers of the striatum and frontal cortex, the thalamus, mesencephalic nucleus, the superior colliculus, inferior colliculus, and most of the cerebellum (Fig. 1).

In the infected areas the majority of positive cells were morphologically identifiable as neurons. Silver grains were deposited predominantly over the cytoplasm consistent with the mode of replication of this virus. Projections from the neuronal cell body were also positive; in many cases, these were clearly identified by their branching structure as dendrites. This was particularly striking in hippocampal neurons, where the dendritic projections of these cells into the molecular layer were clearly outlined by silver grains (Figs. 3D and 4D). Pycnotic nuclei were present in many of the areas of infection (Fig. 3D).

There was minimal involvement of the white matter except for scattered positive cells in the tracts in the deep layers of the striate cortex (Fig. 3C). These cells could be either neuronal or glial. To date we have not been able to identify them by double labeling techniques. In addition, occasional lines of signal were observed which appeared to be infected axons weaving

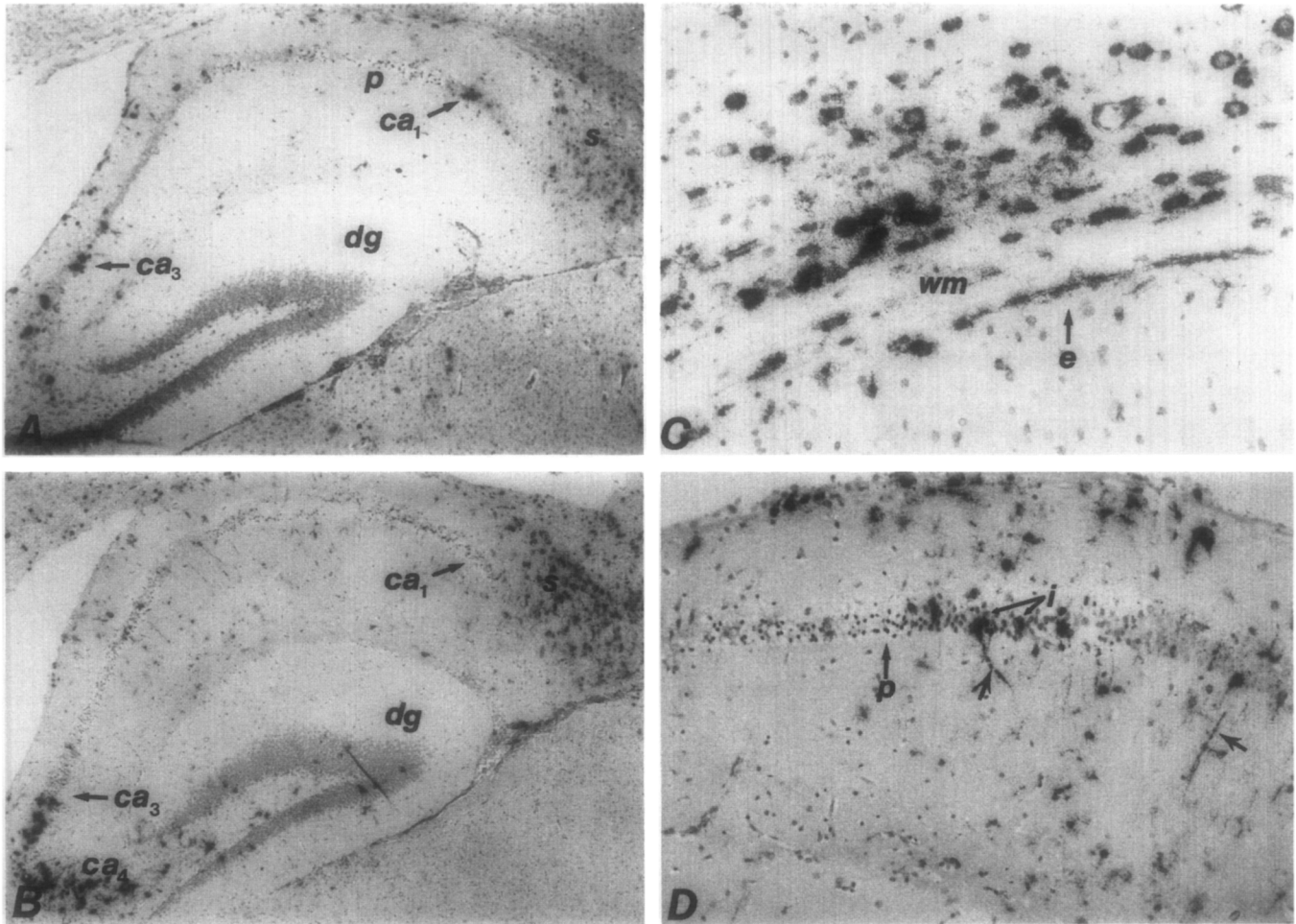


FIG. 3. MHV-4 infection and destruction of cells in the hippocampus. (A) Evidence of infection of neurons in the subiculum: groups of neurons in the ca1 (arrowed) and ca3 areas (arrowed); adjacent are pycnotic nuclei (p) and intact neuronal nuclei. The dentate gyrus (dg) is not involved; 2 days postinfection. (B) Hippocampus infection with the same areas marked as in (A). Note infection in ca4 region of hippocampus and extensive involvement of subiculum; 3 days postinfection. (C) Infected neurons in deep cortical layers above hippocampus. Note spread of infection to endothelial cells of blood vessels (e). A few cells in the white matter tract (wm) are infected; 3 days postinfection. (D) Higher magnification of the ca1 region of the hippocampus shown in (B). Note infected (i) and pycnotic (p) neurons. Dendrites, outlined by silver grains, are also apparent (arrows).

in and out of the plane of the section. Infection of choroid plexi was rare as was infection of endothelial cells. The latter was also unusual and was observed only in a few areas where the vessel or capillary was surrounded by many infected neurons (Fig. 3C). Infection of endothelial cells in areas with few infected neurons was never observed, suggesting that infection of these cells was secondary to infection of the surrounding cells and not vice versa. Ependymal cells were occasionally infected (Fig. 4C).

By 3 days postinfection the overall distribution of virus had not changed but the infection had spread to more cells in each area. This was particularly apparent in the anterior olfactory region, the retrosplenic cortex, the subiculum (Fig. 4A), the vestibular (Fig. 4E) and arcuate nuclei, and in the hippocampal neurons in layers CA1, CA2, and CA3 (compare Fig. 3A, the hip-

poampus of an animal on Day 2 with Fig. 3B, the same area at 3 days). Neuronal destruction was apparent in all these areas. In some areas, the tissue was vesiculated and positive neurons were scattered among predominantly pycnotic cells, with few intact uninfected cells remaining (Figs. 4E and 4F). Involvement of the gray matter of the spinal cord was observed in all mice by Day 3 (Figs. 2A and 2B) but neuronal destruction was not apparent.

An inflammatory response was evident as a low-level meningitis 2 days postinfection and was more intense by 3 days. No *in situ* signal was observed over polymorphonuclear cells, but strong signal was present over occasional mononuclear cells and over clumps of nuclei that appeared to be fused mononuclear cells. These were distributed throughout the meninges. Occasional inflammatory cells were observed around

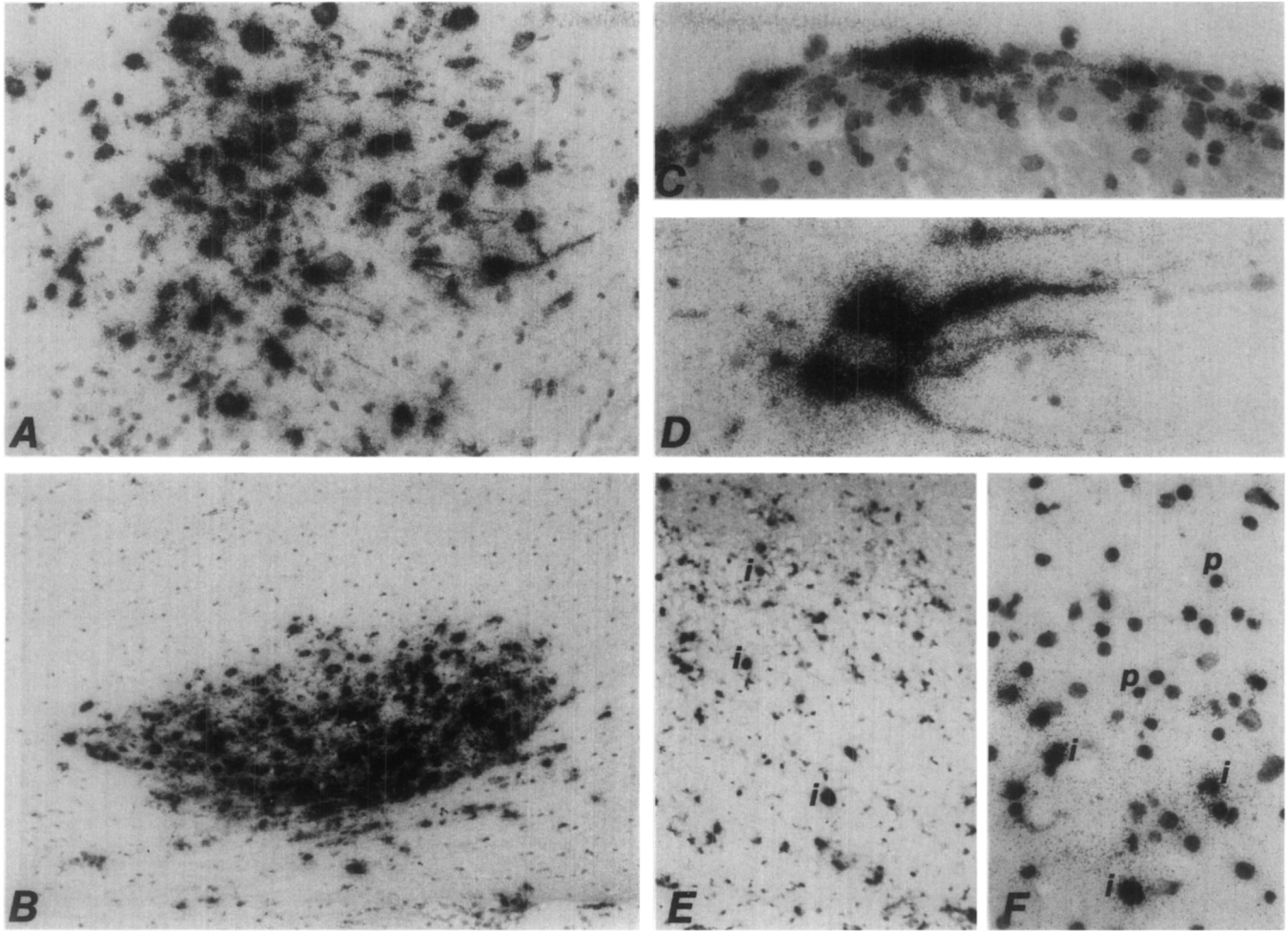


FIG. 4. Pattern of wild-type MHV-4 CNS distribution as detected by *in situ* hybridization in brains of animals infected intracerebrally with MHV-4. (A) Infected neurons in the subiculum; 3 days postinfection. (B) Infection of neurons in a nucleus in the postventral hypothalamus, presumably the supermamillary nucleus. Note that only this nucleus and not cells in the surrounding areas are infected; 3 days postinfection. (C) Infection of ependymal cells; 3 days postinfection. (D) Infection of two adjacent ca1 hippocampal neurons; 2 days postinfection. (E) Destruction of the vestibular nucleus in which a few infected (i) scattered cells remain; 3 days postinfection. (F) Infected (i) and pycnotic cells (p) in the cortex; 4 days postinfection.

some vessels within the brain by Day 3. By Day 4, perivascular cuffing was more widespread and the numbers of inflammatory cells in the cuffs were greater. These were present, in both infected and uninfected areas. In the brain, erythrocytes were present in the areas of destruction and in the ventricles indicating hemorrhaging. Occasional inflammatory mononuclear cells were present in the necrotic areas.

V5A13.1 infection

As illustrated in Fig. 1, the distribution of V5A13.1 in the CNS was very similar to that of the wt virus, except that the variant spread at a much slower rate resulting in less extensive involvement of the same areas (compare Figs. 3B and 5A). As with the wt virus, the principal cell types infected were neuronal. In addition, many

ependymal cells were infected and signal appeared to spread from these cells to underlying cells (Fig. 5B). The endothelial cells of the cerebral capillaries were rarely infected and infection of choroid plexi was never observed. The outer layers of the cortex, the thalamic nuclei, and the cerebellum remained predominantly uninfected.

With the wt virus, signal was first apparent in neurons in the deep layers of the striate cortex with a few positive cells in the underlying white matter tracts of and originating from the corpus callosum (Fig. 3C). This situation was reversed in the case of the variant with few cells infected in the deep cortical layer but many positive cells in the underlying white matter tracts (Figs. 5A, 5C, and 5D). Infection of cells in these tracts was accompanied by destruction. By Day 3 many pycnotic cells were present, the tissue was vesic-

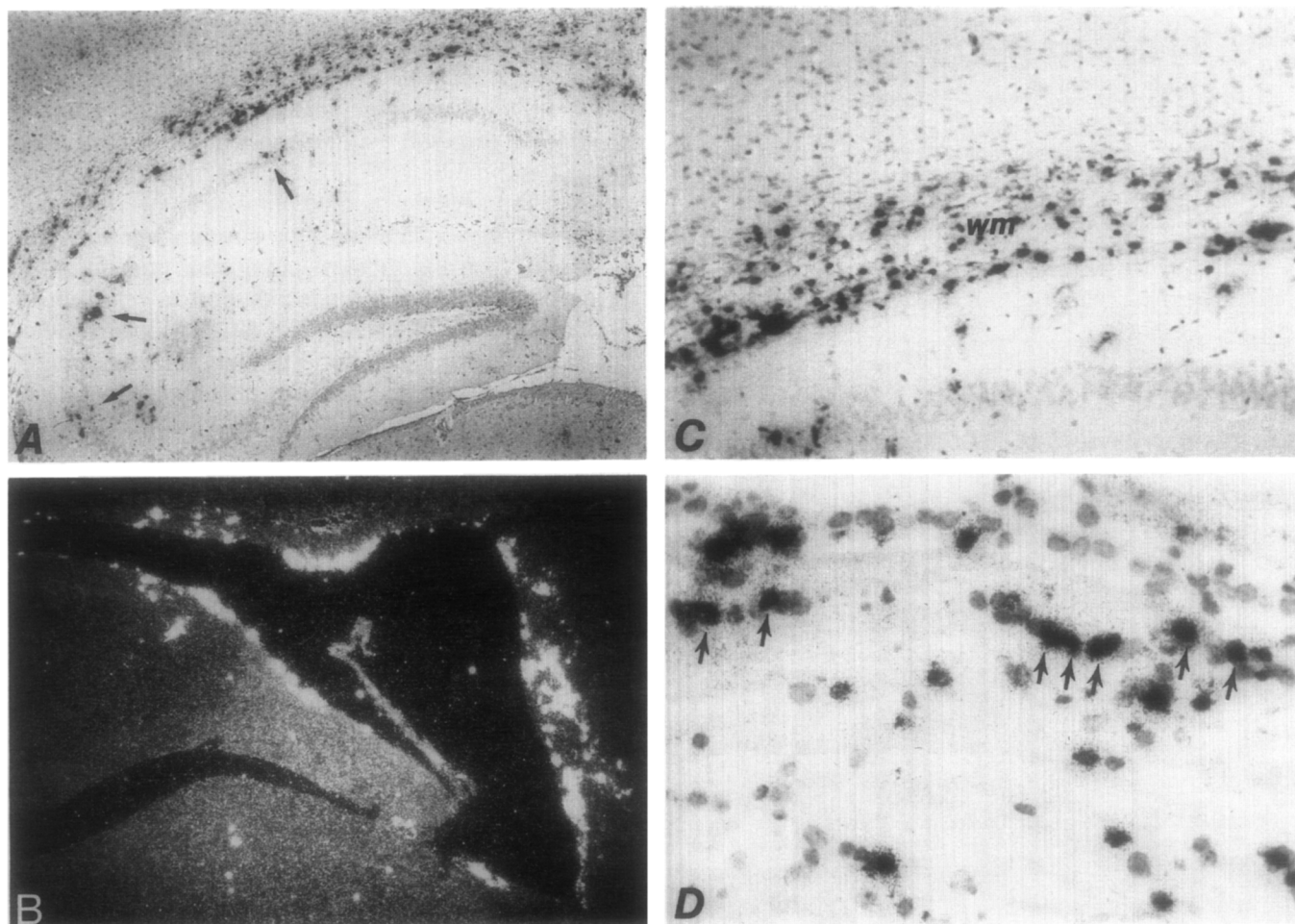


FIG. 5. Pattern of viral nucleic acid distribution as detected by *in situ* hybridization in the brains of mice infected intracerebrally with 100 PFU of V5A13.1; 3 days postinfection. (A) Occasional infection of hippocampal neurons (arrowed), cf, Fig. 3B, showing MHV-4 (cut) infection of the hippocampus at the same time, 3 days postinfection. (B) Dark field image showing positive staining of ependymal cells around the lateral ventricle. Note spread of infection into the underlying parenchyma. (C) Higher magnification of corpus callosum shown in panel A. Note that the infection is of cells in this white matter (wm) tract. In contrast MHV-4 (wt) predominantly infects cells in the overlying deep cortical layers. (D) Higher magnification of corpus callosum shown in (C). Note that some of the infected cells form chains of cells (arrowed), a distribution characteristic of oligodendrocytes.

ulated, erythrocytes were present in many areas, and occasional inflammatory cells were observed.

No virus was detectable in the brain by infectivity assay at Day 7 postinfection (Table 1) but viral RNA was still detected in several areas of the brain after this time by *in situ* hybridization.

Course of infection in athymic *nu/nu* mice

To observe the spread of the infection in the absence of an intact immune system, the V5A13.1 virus was inoculated intracerebrally into athymic *nu/nu* mice. These mice succumbed to intracerebral challenge within 6 days of infection. The distribution of the CNS infection is shown in Fig. 6. Once again neurons were the predominant cell type infected. As with the immunocompetent mice, V5A13.1 spread slowly in the

brains of *nu/nu* mice (compare Figs. 1 and 6). However, in the absence of an intact immune response, the extent of the variant virus infection in the *nu/nu* mice eventually reached a distribution similar to that of wt virus in immunocompetent mice (compare Fig. 1, Day 3 to Fig. 6, Day 4).

DISCUSSION

Mice infected intracerebrally with wild-type MHV-4 develop a fulminant encephalitis which usually results in death by 3 days postinoculation. In contrast, the CNS disease resulting from intracerebral challenge with the V5A13.1 antibody resistant variant of MHV-4 is highly attenuated and requires several logs ($>10^5$) more virus to produce an acute, lethal encephalitis. By localization of MHV-4 and V5A13.1 nucleic acid in the

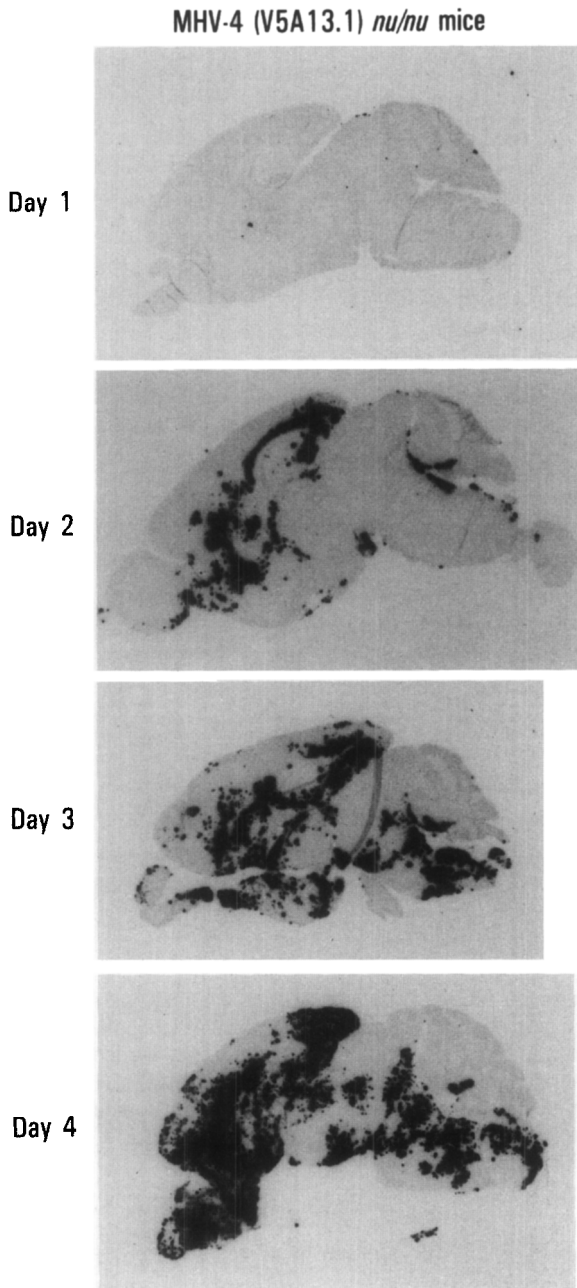


FIG. 6. Distribution of MHV nucleic acid detected by *in situ* hybridization in sagittal sections of brains from *nu/nu* mice infected intracerebrally with 100 PFU of V5A13.1. Tissues are 5- μ m paraffin sections and images were photographed from cronex film. The majority of *nu/nu* mice infected with the variant virus die by Day 6 postinfection.

brains of infected animals we have been able to study the progression of these CNS infections.

The infection of ependymal cells, observed with both viruses although to a different extent, suggests spread of virus via the cerebrospinal fluid. This was also apparent in the spinal cord where the signal radiated out into the gray matter around the spinal canal (Fig. 1). No indication of infection from the blood across cerebral

capillaries was evident. Spread of infection along neuronal pathways was clearly evident in two systems. Firstly, pyramidal cells in the deep cortical layers close to the site of inoculation were among the first cells to be infected followed by their connections in the basal ganglia and caudate (Fig. 1). In this regard it is likely that the consistently infected nucleus in the post-ventral hypothalamus is indeed the supramammillary nucleus since this also receives projections from neurons in the deep layers of the occipital cortex. This would explain the early infection of this distant nucleus. Secondly, tracking along neuronal pathways was apparent in the spread from the anterior olfactory area to the olfactory bulb and out along the olfactory tubercle to connecting nuclei of the rhinencephalon. Spread of virus between adjacent permissive neurons must also occur in order to involve an entire neuronal nucleus (eg., Fig. 4B) and is the likely mechanism of spread along the line of hippocampal neurons (Fig. 3D).

The distribution of MHV in the CNS has previously been investigated in three other systems. An immunohistochemical analysis of the distribution of MHV A59 following ic inoculation demonstrated that this strain of the virus infects neurons in the basal ganglia, particularly in the subthalamic nucleus and the substantia nigra (Fishman *et al.*, 1985). Other areas found to be infected by A59 included neurons of the thalamus, pons, subiculum, regions of the cortex, and glial cells in the corpus callosum. The CNS distribution of A59 virus has also been studied by *in situ* hybridization and electron microscopy following intranasal infection where it was observed that the virus spread from the olfactory into the limbic system (Lavi *et al.*, 1988). Comparison of these results on A59 with the present study indicates many similarities. As with MHV-A59, MHV-4 infected structures in the olfactory and limbic systems, regions of the cortex, and occasional cells in the corpus callosum; however, there were also differences, since the thalamus and substantia nigra were apparent only for their lack of infection by MHV-4. Spread of MHV along defined neuronal pathways has also been observed in a series of studies on the spread and persistence of MHV JHM following intranasal infection of suckling mice (Perlman *et al.*, 1988, 1989). The consistent finding in the present and previous studies is that virus predominantly infects neurons and spread involves tracking along neuronal interconnections. The exact neuronal nuclei involved are likely to vary with the route of inoculation and the strain of the virus and may also be influenced by the age of the animals and the activity of their neuronal pathways.

The major difference between the wt and the variant was not in the neuronal nuclei infected, which were essentially identical, but in the rate at which the viruses spread to and within these nuclei. A subtle difference

in the extent to which the two viruses infected different cell types was also observed. For both viruses the predominantly infected cell type was the neuron, but the variant virus also infected many glial cells. This was most apparent in the deep cortical layers and the underlying white matter. The wt virus infected predominantly neurons in the deep layers of the cortex but spread to involve occasional cells in the adjacent white matter (Figs. 3A and 3C). In contrast, the variant infected many glial cells in the white matter and few neurons in the overlying cortex (Figs. 5A, 5C, and 5D). The variant also appeared to infect more ependymal cells than the wt (Fig. 5B). These effects were not a complete change in the CNS tropism of the variant from that of the wt but represented a change in the degree to which these viruses infected different CNS cell types.

The attenuation of the variant appears not to be related to changes in tropism but to a slower rate of spread (Fig. 1) and consequently reduced neuronal destruction (compare Figs. 3A and 5A). A similar situation has been observed with strains of rabies virus (Coulon *et al.*, 1989). In *nu/nu* mice which lack normal T-lymphocyte functions, the variant virus spread at a slower rate than the wt virus in immunocompetent mice, but by Day 4 the variant in *nu/nu* mice reached a CNS distribution similar to that of the wt virus in immunocompetent mice on Day 3 (compare Figs. 1 and 6). The *nu/nu* mice eventually succumbed to the variant infection around Day 6. Thus it appears that survival of immunocompetent mice infected with the variant virus depends upon intervention by the immune response and control of the infection before it reaches lethal levels. Similarly, a high dose of virus, or a slow immune response can result in death following a V5A13.1 infection, whereas a particularly rapid and effective immune response may offer one explanation for the occasional mouse which survives ic infection with wt virus. The fine balance between viral spread and prevention of viral spread by the immune response is also seen in mice protected from lethal MHV-4 infection by the passive transfer of antibodies (Buchmeier *et al.*, 1984) and in suckling mice inoculated with MHV JHM and nursed on immunized dams (Perlman *et al.*, 1987). Other neuroattenuated strains of MHV may also spread slower in the CNS than the neurovirulent strains. However, this does not appear to be the case with the ts8 mutant of MHV-4 where the basis of the neuroattenuation appears to be a differential cell-tropism (Knobler *et al.*, 1981b).

While we cannot rule out the possibility that differences in neurovirulence between MHV-4 and V5A13.1 lie outside the S gene, this seems to be unlikely given that the selective pressure applied for the generation of the variant virus from the wt was replication in the pres-

ence of a neutralizing monoclonal antibody directed to an epitope on the S glycoprotein (Dalziel *et al.*, 1986). Our sequences of the S gene of plaque purified MHV-4 and its V5A13.1 variant were obtained by direct RNA sequencing of the virus populations (Parker *et al.*, 1989). In the present study we have taken great care to ensure that the viruses inoculated into mice were only one passage from those used for sequencing and that this stock of variant virus maintained the deletion genotype. We conclude that the S1 protein of MHV-4 is likely to contain a determinant (within amino acids 434–575) that directly affects the rate of virus spread within the CNS and resultant neurovirulence. Possible explanations for the reduced rate of viral spread include the following: a reduction in the affinity of the viral envelope glycoprotein for CNS cell surface receptors resulting in a lower efficiency of infection and decreased cell to cell spread; an effect on events that occur subsequent to the virion interaction with cell surface receptors such as liberation of viral nucleic acids into the cytoplasm; or the rate at which the envelope glycoproteins are processed in the assembly of mature infectious virions.

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REFERENCES

- BANNER, L. R., KECK, J. G., and LAI, M. C. (1990). A clustering of RNA recombination sites adjacent to a hypervariable region of the polymerase gene of murine coronavirus. *Virology* **175**, 548–555.
- BAYBUTT, H. N., WEGE, H., CARTER, M. J., and TER MEULEN, V. (1984). Adaptation of coronavirus JHM to persistent infection of murine sac(-) cells. *J. Gen. Virol.* **65**, 915–924.
- BUCHMEIER, M. J., LEWICKI, H. A., TALBOT, P. J., and KNOBLER, R. L. (1984). Murine hepatitis virus-4 (strain JHM)-induced neurologic disease is modulated in vivo by monoclonal antibody. *Virology* **132**, 261–270.
- BUCHMEIER, M. J., DALZIEL, R. G., KOOLEN, M. J., and LAMPERT, P. W. (1987). Molecular determinants of CNS virulence of MHV-4. *Adv. Exp. Med. Biol.* **218**, 287–295.
- COLLINS, A. R., KNOBLER, R. L., POWELL, H., and BUCHMEIER, M. J. (1982). Monoclonal antibodies to murine hepatitis virus-4 (strain JHM) define the viral glycoprotein responsible for attachment and cell-cell fusion. *Virology* **119**, 358–371.
- COULON, P., DERBIN, C., KUCERA, P., PREHAUD, C., and FLAMAND, A. (1989). Invasion of the peripheral nervous system of adult mice by the CVS strain of Rabies virus and its avirulent derivative AvO1. *J. Virol.* **63**, 3550–3554.
- DALZIEL, R. G., LAMPERT, P. W., TALBOT, P. J., and BUCHMEIER, M. J. (1986). Site-specific alteration of murine hepatitis virus type 4 polymerase glycoprotein E2 results in reduced neurovirulence. *J. Virol.* **59**, 463–471.

- ERLICH, S. S., and FLEMING, J. O. (1985). JHM virus infection: A model of viral-induced demyelinating disease. *Curr. Trends Neurosci.* **3**, 276–307.
- FISHMAN, P. S., GASS, J. S., SWOVELAND, P. T., LAVI, E., HIGHKIN, M. K., and WEISS, S. R. (1985). Infection of the basal ganglia by a murine coronavirus. *Science* **229**, 877–879.
- FLEMING, J. O., TROUSDALE, M. D., STOHLMAN, S. A., and WEINER, L. P. (1987). Pathogenic characteristics of neutralization-resistant variants of JHM coronavirus (MHV-4). *Adv. Exp. Med. Biol.* **218**, 333–342.
- GALLAGHER, T. M., PARKER, S. E., and BUCHMEIER, M. J. (1990). Neutralization-resistant variants of a neurotropic coronavirus are generated by deletions within the amino-terminal half of the spike glycoprotein. *J. Virol.* **64**, 731–741.
- HASPEL, M., LAMPERT, P., and OLDSTONE, M. (1978). Temperature sensitive mutants of mouse hepatitis virus produce a high incidence of demyelination. *Proc. Natl. Acad. Sci. USA* **75**, 4033.
- HIRANO, N., MURAKAMI, T., FUJIWARA, K., and MATSUMOTO, M. (1978). Utility of mouse cell line DBT for propagation and assay of mouse hepatitis virus. *Jpn. J. Exp. Med.* **48**, 71–75.
- HIRANO, N., GOTO, N., MAKINO, S., and FUJIWARA, K. (1981). Persistent infection with mouse hepatitis virus JHM strain in DBT cell culture. In "Biochemistry and Biology of Coronaviruses" (V. Ter Meulen, S. Siddell, and H. Wege, Eds.), pp. 301–308. Plenum, New York.
- KNOBLER, R., HASPEL, M., and OLDSTONE, M. (1981a). Mouse hepatitis virus type-4 (JHM strain)-induced fatal central nervous system disease. I. Genetic control and the murine neuron as the susceptible site of disease. *J. Exp. Med.* **153**, 832.
- KNOBLER, R. L., DUBOIS DALCO, M., HASPEL, M. V., CLAYSMITH, A. P., LAMPERT, P. W., and OLDSTONE, M. B. (1981b). Selective localization of wild type and mutant mouse hepatitis virus (JHM strain) antigens in CNS tissue by fluorescence, light and electron microscopy. *J. Neuroimmunol.* **1**, 81–92.
- KNOBLER, R. L., TUNISON, L. A., LAMPERT, P. W., and OLDSTONE, M. B. (1982). Selected mutants of mouse hepatitis virus type 4 (JHM strain) induce different CNS diseases. Pathobiology of disease induced by wild type and mutants ts8 and ts15 in BALB/c and SJL/J mice. *Am. J. Pathol.* **109**, 157–168.
- KOOLEN, M. J., LOVE, S., WOUDE, W., CALAFAT, J., HORZINEK, M. C., and VAN DER ZEIJST, B. A. (1987). Induction of demyelination by a temperature-sensitive mutant of the coronavirus MHV-A59 is associated with restriction of viral replication in the brain. *J. Gen. Virol.* **68**, 703–714.
- KOOLEN, M. J. M., OSTERHAUS, A. D. M. E., VAN STEENIS, G., HORZINEK, M. C., and VAN DER ZEIJST, B. A. M. (1983). Temperature-sensitive mutants of mouse hepatitis virus strain A59: Isolation, characterization and neuropathogenic properties. *Virology* **125**, 393–402.
- LAI, M. M. C., MAKINO, S., SOE, L. H., SHIEH, C. K., KECK, J. G., and FLEMING, J. O. (1987). Coronaviruses: A jumping RNA transcription. *Cold Spring Harbor Symp. Quant. Biol.* **52**, 359–365.
- LAI, M. C. (1990). Coronavirus: Organization, replication and expression of genome. *Annu. Rev. Microbiol.* **44**, 303–333.
- LAMPERT, P., SIMMS, J., and KNIAZEFF, A. (1973). Mechanism of demyelination in JHM virus encephalomyelitis. Electron microscopic studies. *Acta Neuropathol.* [Suppl.] **24**(Suppl.) 76.
- LAVI, E., GILDEN, D. H., HIGHKIN, M. K., and WEISS, S. R. (1986). The organ tropism of mouse hepatitis virus A59 in mice is dependent on dose and route of inoculation. *Lab. Anim. Sci.* **36**, 130–135.
- LAVI, E., FISHMAN, P. S., HIGHKIN, M. K., and WEISS, S. R. (1988). Limbic encephalitis after inhalation of a murine coronavirus. *Lab. Invest.* **58**, 31–36.
- LUYTJES, W., STURMAN, L., BREDENBEEK, P., et al. (1987). Primary structure of the glycoprotein E2 of mouse hepatitis virus strain A59 and identification of the trypsin cleavage site. *Virology* **161**, 479–487.
- MORRIS, V. L., TIESZER, C., MICKINNON, J., and PERCY, D. (1989). Characterization of coronavirus JHM variants isolated from Wistar-Furth rats with a viral-induced demyelinating disease. *Virology* **169**, 127–136.
- PARKER, S. E., GALLAGHER, T. M., and BUCHMEIER, M. J. (1989). Sequence analysis reveals extensive polymorphism and evidence of deletions within the E2 glycoprotein gene of several strains of murine hepatitis virus. *Virology* **173**, 664–673.
- PERLMAN, S., SCHELPER, R., BOLGER, E., and RIES, D. (1987). Late onset, symptomatic, demyelinating encephalomyelitis in mice infected with MHV-JHM in the presence of maternal antibody. *Micro. Pathol.* **2**, 185–194.
- PERLMAN, S., JACOBSEN, G., and MOORE, S. (1988). Regional localization of virus in the central nervous system of mice persistently infected with murine coronavirus JHM. *Virology* **166**, 328–338.
- PERLMAN, S., JACOBSEN, G., and AFIFI, A. (1989). Spread of a neurotropic murine coronavirus into the CNS via the trigeminal and olfactory nerves. *Virology* **170**, 556–560.
- REYNOLDS-KOHLER, C., and NELSON, J. A. (1990). Techniques for double-labelling virus infected cells. In "Viral Pathogenesis: A Practical Approach." (M. B. A. Oldstone, Ed.). IRL Press, Oxford.
- ROBB, J. A., BOND, C. W., and LEIBOWITZ, J. L. (1979). Pathogenic murine coronaviruses. III. Biological and biochemical characterization of temperature-sensitive mutants of JHMV. *Virology* **94**, 385–399.
- SCHMIDT, I., SKINNER, M., and SIDDELL, S. (1987). Nucleotide sequence of the gene encoding the surface projection glycoprotein of coronavirus MHV-JHM. *J. Gen. Virol.* **68**, 47–56.
- SIDDELL, S., WEGE, H., and TER MEULEN, V. (1983). The biology of coronaviruses. *J. Gen. Virol.* **64**, 761–776.
- STOHLMAN, S. A., and FRELINGER, J. A. (1978). Resistance to fatal central nervous system disease by mouse hepatitis virus, strain JHM. I. Genetic analysis. *Immunogenetics* **6**, 277–281.
- STURMAN, L. S., RICARD, C. S., and HOLMES, K. V. (1985). Proteolytic cleavage of the E2 glycoprotein of murine coronavirus: Activation of cell fusing activity by trypsin and separation of two different 90K cleavage fragments. *J. Virol.* **56**, 904–911.
- TAGUCHI, F., SIDDELL, S., WEGE, H., and TER MEULEN, V. (1985). Characterization of a variant virus selected in rat brains after infection by coronavirus mouse hepatitis virus JHM. *J. Virol.* **54**, 429–435.
- TAGUCHI, F., MASSA, P. T., and TER MEULEN, V. C. (1986). Characterization of a variant virus isolated from neural cell culture after infection of mouse coronavirus JHM. *Virology* **155**, 267–270.
- WEGE, H., KOGA, M., WATANABE, R., NAGASHIMA, K., and TER MEULEN, V. (1983). Neurovirulence of murine coronavirus JHM temperature-sensitive mutants in rats. *Infect. Immun.* **39**, 1316–1324.
- WEGE, H., MASSA, P. T., DORRIES, R., WATANABE, R., and TER MEULEN, V. (1986). Coronavirus infection of rats: a model for virus induced demyelinating disease associated with autoimmunity. In "Modern Trends in Virology" (H. Bauer, Ed.) Springer Verlag, New York.
- WEGE, H., WINTER, J., and MEYERMANN, R. (1988). The peplomer protein E2 of coronavirus JHM as a determinant of neurovirulence: Definition of critical epitopes by variant analysis. *J. Gen. Virol.* **69**, 87–98.