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Site-Specific Alteration of Murine Hepatitis Virus Type 4 Peplomer Glycoprotein E2 Results in Reduced Neurovirulence†

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Strains of the murine coronavirus mouse hepatitis virus type 4 (MHV-4) which contained a mutation in the E2 peplomer glycoprotein were obtained by selection for resistance to neutralization by monoclonal antibodies. Characterization of six variants representing two independent epitopes on E2, E2B and E2C, by in vitro neutralization and antibody-binding assays demonstrated that selection for an alteration in epitope E2B also resulted in changes in epitope E2C and vice versa. We observed a mutation frequency of approximately $10^{-4.3}$ to $10^{-4.6}$, which is consistent with the expected occurrence of single point mutations. The variant virus strains were attenuated with respect to neurovirulence when compared with wild-type MHV-4. Mice normally develop encephalomyelitis and die after wild-type MHV-4 infection. Mice receiving 2- to 3-log-higher doses of the variant strains survived and developed demyelinating disease. As the disease progressed, evidence of remyelination and ongoing demyelination was observed up to 65 days after infection. Virus reisolated 15 days after infection retained the variant phenotype. The data indicate that the E2 glycoprotein plays a central role in determining the cellular tropism and virulence of MHV-4 in the mouse.

Murine hepatitis virus type 4 (MHV-4; JHM strain) (1) is a neurotropic member of the *Coronaviridae* (for a review, see reference 33). Infection of adult mice by MHV-4 usually culminates in encephalomyelitis with over 95% mortality. In the small fraction of mice which survive the initial infection by wild-type virus, chronic white matter disease occurs which is characterized by focal demyelinating lesions in the brain and spinal cord with subsequent remyelination and recurrent demyelination (15, 16). The development of demyelinating lesions is thought to be a primary effect of virus infection and destruction of the oligodendrocyte (16, 34), and virus has been shown in one instance to persist in the infected brain for up to 1 year after infection (15). Resistance to MHV-4 infection in certain mouse strains, notably SJL/J, is controlled by a single autosomal recessive gene on chromosome 7 (14). Resistance is expressed in vivo and in vitro in both neurons and macrophages. In the latter cell type cultured in vitro, it appears that virus is able to establish a primary infection in a limited number of cells of the resistant genotype but is unable to spread to adjacent cells in the culture (13). Several groups have described temperature-sensitive (*ts*) or spontaneously occurring plaque morphology mutants of MHV-4 which are altered in their pathogenicity; this is in marked contrast to the highly neurovirulent wild-type MHV-4. Haspel et al. (11) isolated a mutant referred to as *ts8* which upon intracerebral (i.c.) inoculation causes no demonstrable encephalitis but induces extensive white matter lesions. Studies of this mutant demonstrated primary destruction of oligodendroglia with little evidence of inflammatory response or neuronal involvement. Stohlman et al. (25) described similar disease induced by a spontaneously arising plaque morphology mutant termed

DS. In neither model is a specific genetic lesion in the mutant virus associated with loss of neuropathogenicity.

MHV-4 contains three major structural polypeptides, a nucleocapsid protein and two glycoproteins, E1 and E2 (12, 27, 32). By using monoclonal antibodies (MAbs) directed against these MHV-4 polypeptides, Collins et al. (6) demonstrated that only anti-E2 MAbs neutralize virus infectivity in vitro and concluded that the viral structure bearing active sites for viral attachment to host cells and for cell-to-cell fusion is the 180-kilodalton E2 glycoprotein. Cleavage of E2 to two nonidentical 90-kilodalton subunits is required for activation of the fusion potential (28). Talbot et al. (30, 31) identified three neutralizing epitopes (E2A, E2B, and E2C) and two nonneutralizing epitopes (E2D and E2E) on E2. Passively transferred MAbs directed against epitopes E2A and E2B protect mice from lethal challenge with MHV-4 (3). In protected mice, MHV-4 infection is not blocked, but virus grows to titers 20-fold lower than those in control, unprotected mice. Less extensive neuronal involvement is observed, and no spread to neurons in the spinal cord occurs. Oligodendrocytes in the spinal cord are infected, and demyelination is evident in the protected mice. Moreover, the lesions in these mice are associated with mild to moderate mononuclear infiltrates (3). These data show that MAbs to specific determinants on the E2 glycoprotein are capable of altering the tropism and course of MHV-4 infection from fatal encephalitis to a demyelinating disease. To further investigate the role of E2 in disease, we used MAbs to select variant virus strains which resisted neutralization. The i.c. infection of mice with these E2 variants resulted in the development of primary demyelination, with encephalitis occurring only at virus doses 2 to 3 orders of magnitude higher than the fatal wild-type dose. These variants were therefore attenuated with respect to their virulence in mice. Data in this study indicate that the E2 glycoprotein plays a pivotal role in determining the cellular tropism and virulence of MHV-4 in the mouse central nervous system (CNS).

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MATERIALS AND METHODS

Virus and cell culture. MHV-4 (JHM strain) was originally obtained from L. P. Weiner and is routinely propagated on the DBT mouse astrocytoma cell line as described previously (3).

MAbs. MAbs used in this study were either generated in this laboratory (6) or graciously provided by John Fleming of the University of Southern California. The properties of all the MAbs have been described previously (3, 6, 8, 31).

Selection of variant viruses. A volume of 0.5 ml containing 5×10^5 PFU of a stock of MHV-4 that was plaque purified three times was incubated for 30 min at 37°C with a 1:20 dilution of ascites fluid containing either anti-E2 MAb 4B11.6 or anti-E2 MAb 5A13.5. The MAbs were previously shown to have virus neutralization titers of 1:31,600 and 1:158,000, respectively, expressed as 50% plaque reduction doses per ml (3, 31). Virus that escaped neutralization was plated onto DBT cells and cultivated in the presence of MAb (1:40 dilution of ascites fluid) in the overlay medium for 24 h, at which time a few foci of cytopathic effect were observed. Medium was removed, and virus was plaque purified. Picked plaques were suspended in 0.2 ml of medium containing a 1:20 dilution of ascites fluid and incubated at 37°C for 1 h. Virus that escaped round 2 of neutralization was plaque purified twice more, and a working stock was prepared and retested for resistance to neutralization. Three variant virus stocks selected with each MAb were chosen for detailed analysis.

LD₅₀ test. Eight BALB/c Byj mice were inoculated i.c. with the indicated dose of virus in a total volume of 50 µl. The number of mice surviving was recorded each day for 14 days, at which time the 50% lethal dose (LD₅₀) was calculated by the method of Reed and Muench (22).

Virus neutralization. The ability of the variant viruses to escape neutralization was quantitated by a plaque reduction neutralization assay. Ascites fluid was diluted in minimal essential medium as indicated above and mixed with an equal volume (0.5 ml) of virus stock diluted to contain 200 PFU of MHV-4. The virus-MAb mixtures were incubated at 37°C for 30 min, divided in half, and plated in duplicate on monolayers of DBT cells in 35-mm tissue culture wells. After adsorption for 1 h, monolayers were overlaid with 0.5% agarose in medium 199 supplemented with 10% fetal bovine serum and incubated for a further 72 h in a 37°C CO₂ incubator. Cells were then fixed by the addition of 2 ml of 25% Formalin in phosphate-buffered saline (PBS) for 4 to 18 h. Overlays were removed after fixation, and monolayers were stained with 0.1% crystal violet. Plaques were counted, and percent neutralization was calculated.

Histopathology. For histology, anesthetized mice were sacrificed by perfusion via the left ventricle with cold PBS followed by 2.5% glutaraldehyde in PBS. Spinal cords and brains were removed and placed in 2.5% glutaraldehyde overnight, after which the tissue samples were transferred to PBS and stored at 4°C. Tissues were embedded in Epon and stained with hematoxylin and eosin for histology or with *para*-phenylenediamine for detection of myelin. For electron microscopy, thin sections were stained with uranyl acetate and lead citrate and examined with a Siemens 101 electron microscope.

Cocultivation. Brains were removed aseptically and diced into 2- to 3-mm fragments. Trypsin EDTA solution (20 ml; GIBCO Laboratories) was added, and the suspension was gently rotated for 15 min at 22°C. Large fragments of tissue were allowed to settle at $1 \times g$, and the supernatant was

decanted. More trypsin EDTA solution (20 ml) was added, and the digestion procedure was repeated. The supernatants were pooled, and the cells were pelleted. The cell pellets were suspended in 1 ml of minimal essential medium and plated onto 80% confluent monolayers of DBT cells. Medium was replaced after 48 h, and the cocultures were screened daily for the appearance of syncytia. Virus was recovered from the supernatant of frozen and thawed cocultures by passage on DBT cells. In some instances early in infection, virus was detected by plaque assay in 10% (wt/vol) homogenates of brain and liver.

Antibody-binding assay. The binding of anti-E2 MAbs to variant or wild-type MHV-4 was assayed by indirect immunofluorescence on acetone-fixed infected cell monolayers. MAb culture fluids were used undiluted, and staining was developed by using fluorescein isothiocyanate-conjugated sheep anti-mouse immunoglobulin G prepared in this laboratory (4, 30).

RESULTS

Selection of MAb-resistant variants. A 5×10^5 -PFU sample (in 0.5 ml) of a stock of MHV-4 that had been plaque purified three times was neutralized by incubation for 30 min at 37°C with a 1:20 dilution of ascites fluid containing either MAb 5A13.5 or MAb 4B11.6. Approximately 0.01 to 0.1% of the virus escaped neutralization, and of this resistant virus, approximately 10% of the clones were bona fide MAb-resistant variants (data not shown). Variants obtained in this manner were assayed for resistance to neutralization by MAbs directed against epitopes E2A, E2B, and E2C. A total of 12 variants were selected by MAb 5A13.5, and 24 variants were selected by MAb 4B11.6. Variants were selected by these MAbs at mutation frequencies of $10^{-4.6}$ and $10^{-4.3}$, respectively. Variants obtained by using MAb 5A13.5 were designated V5A13.1, V5A13.2, V5A13.3, etc., those selected with MAb 4B11.6 were designated V4B11.1, V4B11.2, V4B11.3, etc.

Neutralization of variant and wild-type MHV-4 by MAbs. The ability of MAbs 5B19.2 (E2A), 5A13.5 (E2B), and 4B11.6 (E2C) to neutralize wild-type MHV-4 or the variants was determined. All three MAbs neutralized over 99% of wild-type virus effectively at low dilutions (i.e., 1:100) and had titers of $10^{3.5}$ (5B19.2), $>10^{4.3}$ (5A13.5), and $10^{4.2}$ (4B11.6) (Fig. 1). Variants V5A13.1, V5A13.2, and V5A13.3 resisted neutralization by MAb 5A13.5 (the selecting MAb) and by MAb 4B11.6 but did not resist neutralization by MAb 5B19.2. In like manner, variants V4B11.1, V4B11.2, and V4B11.3 resisted neutralization by both MAbs 5A13.5 and 4B11.6. Therefore, it appears that a mutation resulting in acquisition of resistance to MAbs against epitope E2B also confirmed resistance at epitope E2C and vice versa.

Binding of anti-E2 MAbs to the variant viruses. Resistance to neutralization may result either from the failure of MAb to bind viral antigen or from the inability of the bound MAb to induce structural perturbations or block functional sites required for successful infection. To investigate these alternatives, we assessed the binding of 17 anti-E2 MAbs to wild-type MHV-4, to the *ts8* mutant (11), and to six MAb-resistant variants. Representative results are shown in Fig. 2 and are summarized in Table 1. All 17 MAbs bound to both wild-type MHV-4- and to *ts8*-infected cells. MAbs to epitopes E2A, E2D, and E2E (30, 31) as well as several MAbs from J. Fleming, which have not been assigned to any of the epitopes described by Talbot et al. (31), also bound to all the variant strains, indicating that the epitopes recognized

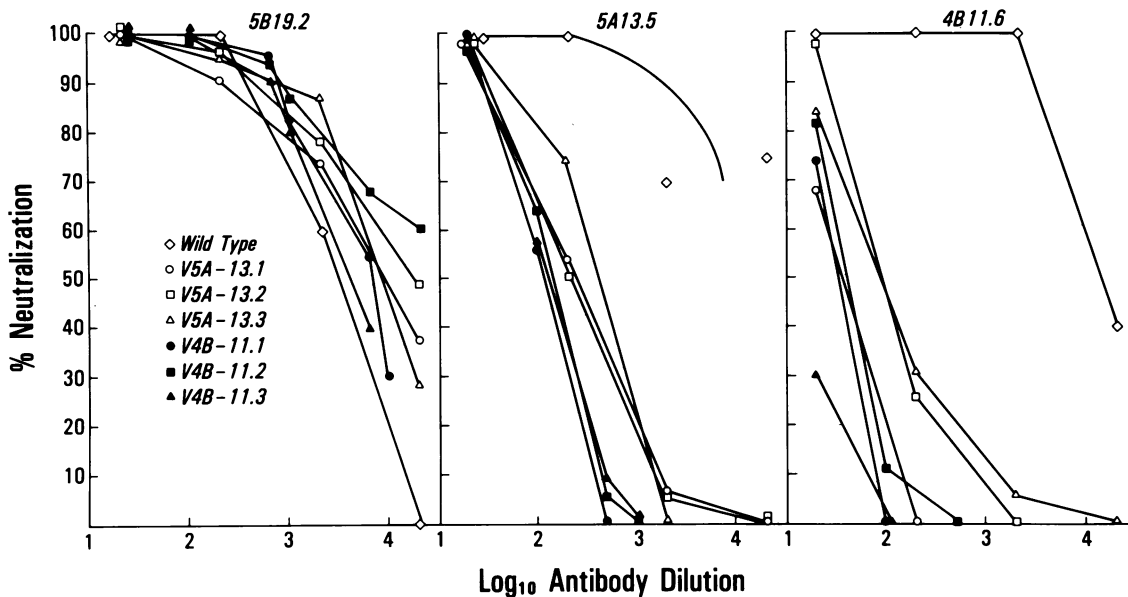


FIG. 1. Neutralization of wild-type and MAb-selected variant strains of MHV-4 by anti-E2 MAbs. MAbs to epitopes E2A (5B19.2), E2B (5A13.5), and E2C (4B11.6) were assayed over a 4-log range for the ability to neutralize wild-type MHV-4 (JHM) and the six variants V5A13.1, V5A13.2, V5A13.3, V4B11.1, V4B11.2, and V4B11.3. All seven virus strains were effectively neutralized by MAb 5B19.2 (epitope E2A). Variants V5A13.1, V5A13.2, and V5A13.3 were resistant to neutralization by MAbs 5A13.5 (epitope E2B, the selecting MAb) and 4B11.6 (epitope E2C). Similarly, variants V4B11.1, V4B11.2, and V4B11.3 resisted neutralization by both MAbs 5A13.5 and 4B11.6.

by these MAbs were intact on the variant E2 polypeptides. MAbs 5A13.5 (epitope E2B) and 4B11.6 (epitope E2C) failed to bind to any of the variants, confirming data obtained by neutralization assay. Several unassigned MAbs also failed to

bind to variant E2 polypeptides. At least two of these, J.7.5 and J.7.6 (8) are thought to recognize structures different from those recognized by MAbs 5A13.5 and 4B11.6 (30). This result strongly suggests that the alteration in the variant

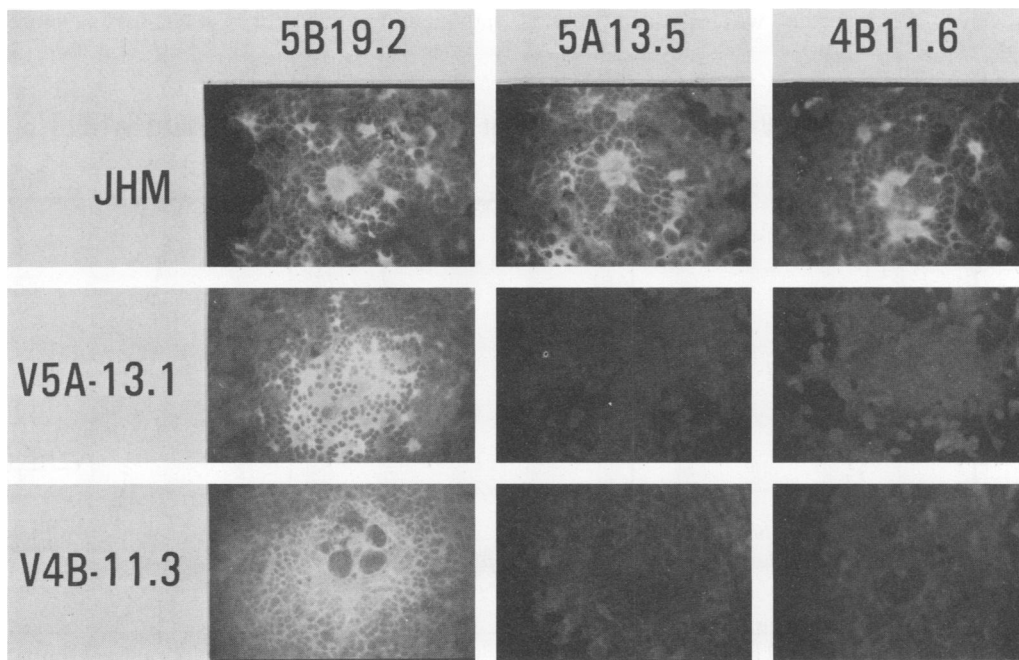


FIG. 2. Binding of anti-E2 MAbs to wild-type and MAb-selected variant strains of MHV-4. DBT cell monolayers infected with wild-type MHV-4 (JHM) or the variants V5A13.1 or V4B11.3 were acetone fixed at 18 h postinfection. The ability of MAbs 5B19.2 (epitope E2A), 5A13.5 (epitope E2B), and 4B11.6 (epitope E2C) to bind to viral antigen was determined by indirect immunofluorescence. Bound MAb was visualized by using fluorescein isothiocyanate-conjugated sheep anti-mouse immunoglobulin G. MAb 5B19.2 bound to wild-type MHV-4 (JHM) and both variants. MAbs 5A13.5 and 4B11.6 also bound to wild-type MHV-4 (JHM) but failed to bind to either variant.

TABLE 1. Reactions of variant and wild-type MHV-4 strains with anti-E2 MAbs^a

MAb	Neutralization	Virus strain					
		V5A13.1	V5A13.2	V5A13.3	V4B11.1	V4B11.2	V4B11.3
5B19.2	+	+	+	+	+	+	+
5B170.3	+	+	+	+	+	+	+
5A13.5	+	-	-	-	-	-	-
4B11.6	+	-	-	-	-	-	-
5B21.5	-	+	+	+	+	+	+
5B93.7	-	+	+	+	+	+	+
5B207.3	-	+	+	+	+	+	+
5B216.8	-	+	+	+	+	+	+
J.1.2	-	-	+	+	+	+	-
J.71.8	+	-	-	-	-	-	-
J.2.2	+	-	-	-	-	-	-
J.7.2	+	-	-	-	-	-	-
J.2.5	-	+	+	+	±	±	+
J.2.6	+	+	+	+	+	+	+
J.7.1	-	+	+	+	+	+	+
J.7.5	+	-	-	-	-	-	-
J.7.6	+	-	-	-	-	-	-

^a All 17 MAbs had strong positive reactions to both wild-type MHV-4- and *ts8*-infected cells. +, Strong positive reaction; ±, weak positive reaction; -, no reaction.

E2 polypeptides results in a conformation change(s) which affects several distinct topographic sites on E2. The observation that MAb J.1.2 failed to bind to the variants we selected, V5A13.1 and V4B11.3, yet did bind to the four remaining variants suggests that these are distinct variant strains. Neutralization of the variants we selected by MAb J.1.2 was not tested.

Virulence of variant viruses. BALB/c Byj mice were inoculated i.c. with 50 and 1,000 PFU of each of the variant viruses V5A13.1, V5A13.2, V5A13.3, V4B11.1, V4B11.2, V4B11.3, or with wild-type MHV-4. Infected mice were observed for 14 days, and mortality was recorded. After

inoculation with wild-type MHV-4, all the mice died by day 7. In contrast, inoculation with the variant strains resulted in little mortality, indicating that the variants were attenuated in neurovirulence. Following up these initial observations, we selected variants V5A13.1 and V4B11.3 and determined LD₅₀ values for each (Fig. 3). Wild-type MHV-4 (JHM) had an LD₅₀ of less than 0.45 PFU, confirming the high virulence of that virus. Variants were greatly reduced in pathogenicity in this assay; V5A13.1 had an LD₅₀ of >1,800 PFU, the highest dose we could inoculate safely in 0.05 ml, and V4B11.3 had an LD₅₀ of 95 PFU. Thus, V5A13.1 was attenuated by a factor of 4,000-fold or more, and V4B11.3

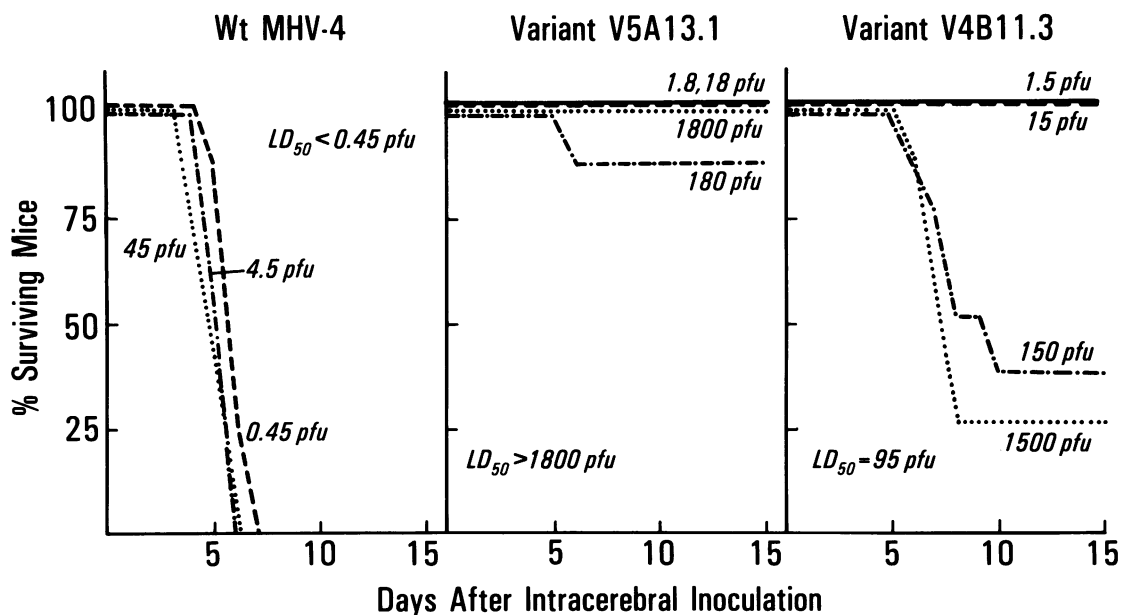


FIG. 3. Attenuation of MAb-selected variants of MHV-4. Eight BALB/c Byj mice were i.c. inoculated with the indicated doses of wild-type MHV-4 (JHM) or variants V5A13.1 and V4B11.3 in a total volume of 50 μ l. The number of mice surviving was recorded each day for 14 days, at which time the LD₅₀ was calculated by the method of Reed and Muench (22).

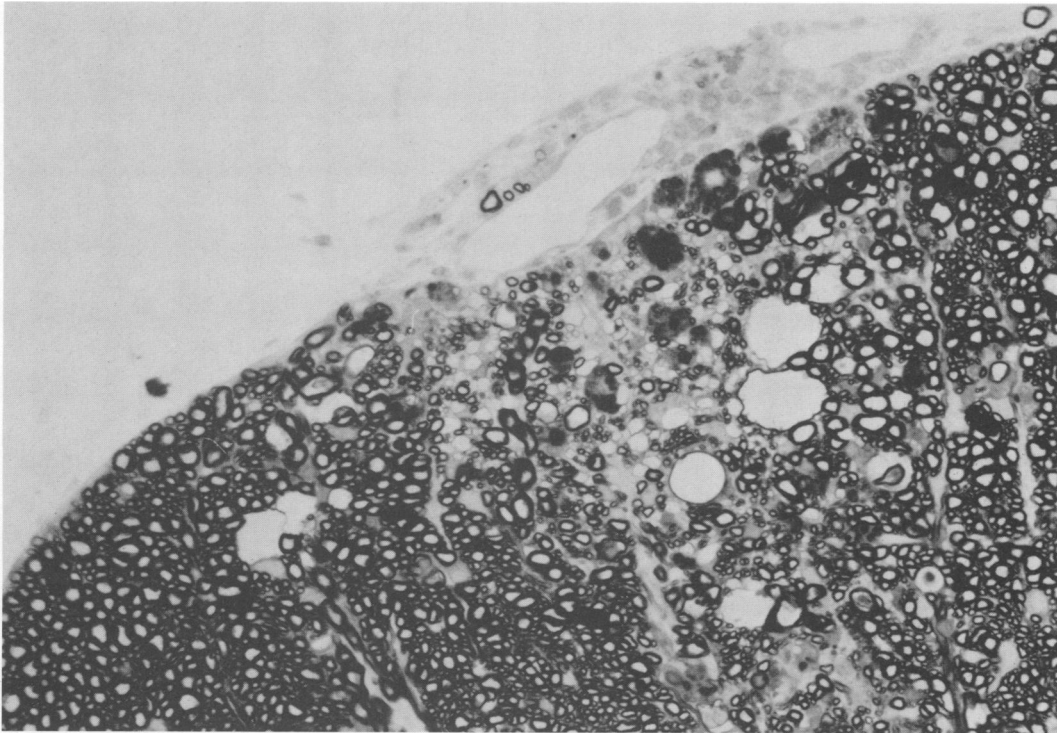


FIG. 4. Focal area of demyelination next to an inflammatory infiltrate in the meninges. Resin-embedded tissue from the spinal cord of a BALB/c mouse at 15 days postinfection. Magnification, $\times 500$.

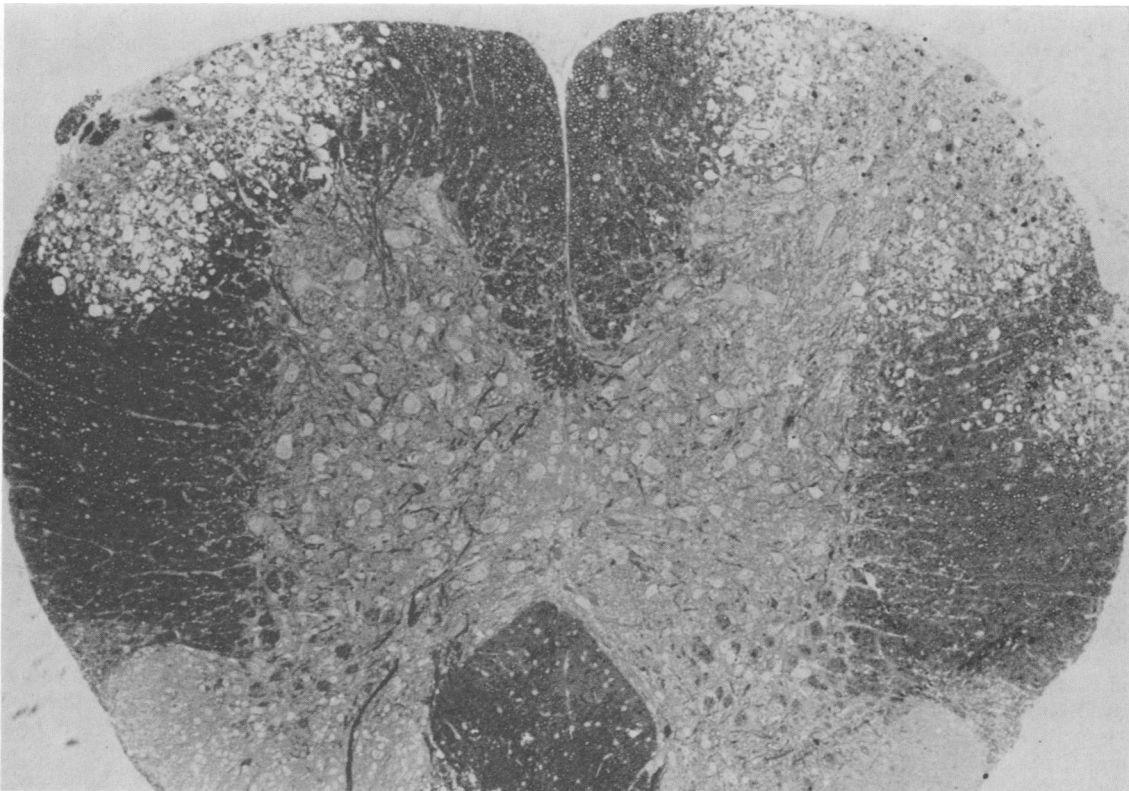


FIG. 5. Plaques of demyelination in the spinal cord of a mouse at 32 days postinfection. Magnification, $\times 50$.

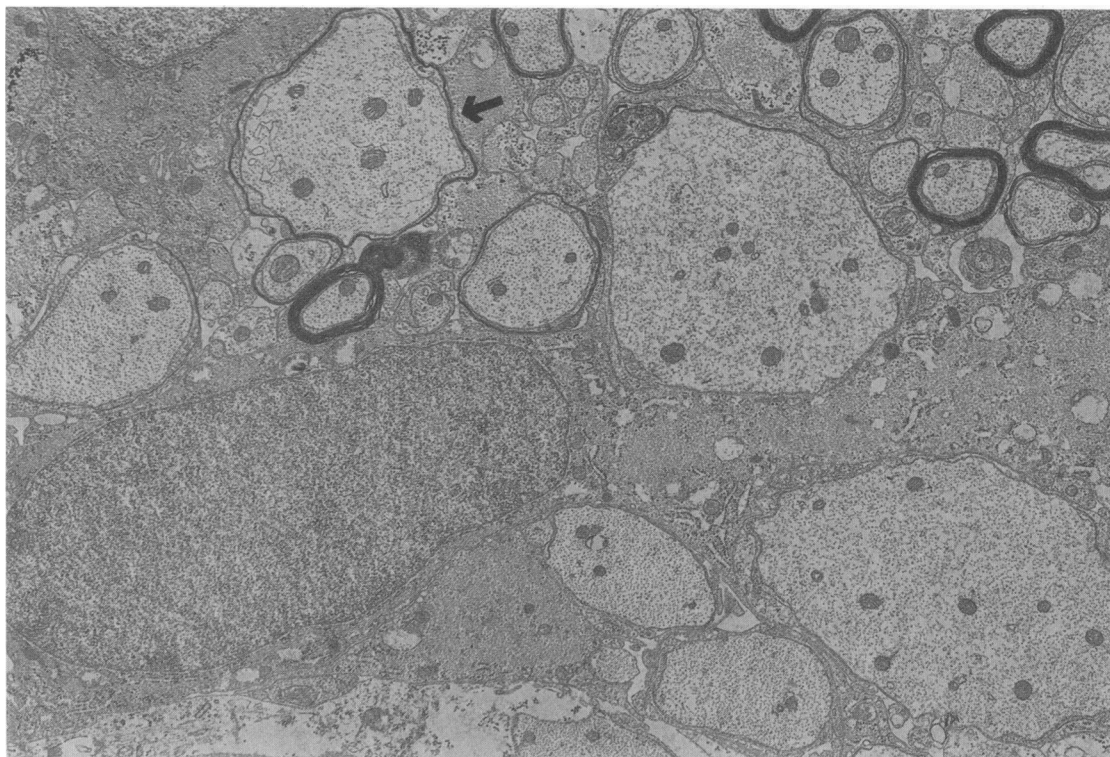


FIG. 6. Demyelination and early remyelination (arrow) in a lesion from the spinal cord at 15 days postinfection. Magnification, $\times 8,000$.

was attenuated by approximately 200-fold relative to wild-type virus.

Histopathological evidence of subclinical disease in variant virus-infected mice. BALB/c Byj mice were inoculated i.c. with 0.5 LD₅₀ of V5A13.1 or V4B11.3 (900 and 50 PFU, respectively), and groups of two mice were killed by cardiac perfusion on days 4, 15, 22, 32, 50, and 65 postinfection. At 4, 15, and 22 days, moderate mononuclear infiltration was observed in the spinal cord with inflammatory cells penetrating into the white matter (Fig. 4). *Para*-phenylenediamine-stained plastic-embedded sections of spinal cord taken 15, 22, 32, 50, and 65 days postinfection showed plaques of demyelination as well as areas of remyelination (Fig. 5 and 6). Examination of these lesions by electron microscopy revealed naked axons. Thin myelin sheaths (Fig. 6), indicative of a remyelinating repair process, were evident as early as 15 days postinfection and persisted for the duration of the study.

Isolation of virus from infected brains by cocultivation. Brains were aseptically removed from one mock-, two V5A13.1- and two V4B11.3-infected mice at days 15, 32, 50, and 65, and virus was cocultivated on DBT cells as described above. We were able to recover and subculture virus at day 15 from a V4B11.3-infected mouse, but attempts at cocultivation of virus from V5A13.1-infected mice were negative. The virus recovered from a V4B11.3-infected mouse was found to be resistant to neutralization by both MAbs 4B11.6 and 5A13.5. Infectious virus was isolated directly from brain and liver homogenates at 4 days postinfection, verifying that virus had infected these mice and had spread to non-CNS sites of replication. A summary of the virus titers in brains and livers at various times postinfection is presented in Table 2.

DISCUSSION

Viral etiology of CNS demyelinating diseases is well established in several animal models, including MHV-4 (15, 16), Theiler's virus (18), and visna virus infections (19). Similarities of pathologic features between these experimentally induced diseases and human disease as well as epidemiologic observations suggesting infectious etiology (5) have led investigators to propose a viral etiology for human multiple sclerosis (35). Factors which affect the course of CNS infections and ultimately determine whether an infection will be acute and self-limited, lethal, or chronic and degenerative are poorly understood. Thus, studies of experimental models such as MHV-4 are likely to yield new insight. In the present report, we focused on molecular determinants of MHV-4 neurotropism. We present evidence indicating that mutations in the MHV-4 E2 peplomer glycoprotein reflected by acquisition of resistance to neutralizing MAbs resulted in attenuation of neurovirulence. Mice which would normally die following wild-type MHV-4 infection survived infection after receiving doses of the MAb-resistant variant viruses 2 to 3 log higher than the wild-type dose. This report and a similar study by J. Fleming and colleagues (9) represent the first direct demonstrations of involvement of the E2 glycoprotein in determination of MHV-4 neurovirulence.

The i.c. inoculation of susceptible mice with wild-type MHV-4 (JHM) normally results in encephalomyelitis and death within 7 days. Underlying the fatal disease is a primary demyelinating condition which is observed in the small percentage of animals which survive the infection or are spared by passive transfer of protective antibody (3).

It was suggested by previous studies that the E2 peplomer

TABLE 2. Virus content in brain and liver at various times after MHV-4 infection^a

Day (mouse no.)	Virus strain					
	V4B-11.3		V5A-13.1		Wild-type MHV-4 (JHM) ^b	
	Brain	Liver	Brain	Liver	Brain	Liver
2						
1	2.5×10^3	<40	3.3×10^4	<40	5×10^4	5×10^2
2	2.5×10^3	2.0×10^2	3.0×10^4	4.0×10^2		
4						
1	3.5×10^4	<40	2.5×10^4	1.0×10^5	1×10^6	1×10^4
2	3.0×10^3	<40	4.0×10^3	<40		
7						
1	<40	<40	<40	<40	4×10^5	3×10^4
2	<40	<40	<40	<40		

^a Determined by plaque titration of 10% brain or liver homogenate. The limit of sensitivity of the plaque titration assay was 40. No virus was detected in variant strain-infected tissues later than day 7.

^b Data from Knobler et al. (15).

glycoprotein of MHV-4 plays a role in determining viral pathogenicity in vitro (6, 26) and in vivo (2, 3, 29). We used MABs against two distinct topographical sites on MHV-4 E2 to select variant virus strains containing a mutation in E2, and we studied the effect of such mutations on virulence. We calculated that these variants arose in the population at a mutation frequency of approximately $10^{-4.3}$ to $10^{-4.6}$, a value which agrees with the predicted mutation frequency for single-stranded RNA viruses of 10^{-4} to 10^{-5} (17, 21), making it improbable that the variants we derived contained additional non-E2 mutations which could have affected pathogenicity.

Characterization of six variant virus strains representing the two independent epitopes E2B and E2C (31) by neutralization and antibody-binding assays demonstrated that selection for MAB resistance at epitope E2B also resulted in changes at epitope E2C. The reciprocal was also observed, i.e., selection for resistance at E2C resulted in changes at E2B. Additional binding studies showed that several MABs which recognize antigenic sites distinct from epitopes E2B and E2C (8, 30) also failed to bind to the E2 variants we selected, indicating that these epitopes were also altered. This observation of simultaneous acquisition of resistance at multiple epitopes can best be reconciled if a point mutation has resulted in conformational changes in the E2 molecule. The fact that topographically distinct epitopes are affected suggests that these sites may be clustered. Previous studies from our laboratory (31) showed that the MABs we used to select variants recognize conformational sites on E2. In contrast to the relative ease with which we isolated mutants affecting epitopes E2B and E2C, we were unable to select mutants with MAB against the sequential epitope E2A (data not shown). Confirmation of the location and nature of the mutation(s) on E2 will require nucleotide sequence and protein structural data which is not currently available.

Disease induced by the variant viruses was characterized by moderate early inflammatory lesions in the brain and spinal cord which subsided between 15 and 22 days, leaving foci of demyelinated axons. As disease progressed, evidence of remyelination and new demyelination was observed. At the termination of the present observations 65 days postinfection, demyelination was extensive. A similar course of primary demyelination was observed after infection with MHV-4 *ts8* (11); however, in that model, inflammation is not a prominent feature of the disease. Koolen et al. (M. J. M.

Koolen, W. Wouda, J. Calafat, M. C. Horzinek, and B. A. M. van der Zeijst, personal communication) reported the isolation of a temperature-sensitive mutant, *ts342*, of the hepato-neurovirulent MHV strain A59. Whereas wild-type A59 virus-infected mice died of hepatitis, *ts342*-infected mice survived and developed chronic demyelinating disease characterized by infection of oligodendrocytes and astrocytes, accompanied by widespread inflammatory reactions in the white matter. These investigators isolated three independent revertants of *ts342* with biological properties indistinguishable from the wild-type A59 virus. Thus it appears that attenuation in this case also results from a single point mutation. The absence of encephalomyelitis after variant virus infection is most probably attributable to a change in viral tropism resulting in sparing of the neurons. Under these conditions, the demyelinating potential of the virus becomes the predominant pathologic feature. Such altered cell tropism was already demonstrated for *ts8*, which infects oligodendrocytes but not neurons (15), and studies are being carried out to characterize the tropism of the variants.

The fate of the virus postinfection remains to be determined. We were unable to reisolate infectious virus later than 15 days postinfection, although a cytopathic effect typical of MHV-4 was seen in cultures cocultivated with brain cells taken 32 days postinfection. This may reflect sequestration of the virus or deficiencies in the methods available for recovery. Nonetheless, we demonstrated that the virus reisolated at 15 days retained the variant phenotype. Observation of moderate inflammatory response in the CNS suggests that the disease may have an immune-mediated component. We are currently assaying tissues taken through the course of infection for viral antigens and RNA in an effort to define the fate of virus during the course of infection.

Similar observations of site-specific alteration of neurovirulence have been made in other virus infections. Inoculation of newborn mice with reovirus type 3 results in acute fatal encephalitis with virus replication occurring in neurons in multiple regions of the cerebral cortex. Spriggs and Fields (24) used neutralizing MAB against the hemagglutinin molecule (σ 1) to select variant viruses which were attenuated with respect to neurovirulence and showed reduced replication in the brains of newborn mice. Infection by these variants was regionally localized in the hippocampus

(23) and failed to spread throughout the CNS. Reduced neurovirulence was traced to altered tropism, resulting in restricted growth in the CNS, and recent studies showed that the site plays a role in binding to cellular receptors on neurons (20).

Similar findings have been documented in rhabdoviruses and bunyaviruses. Variants of rabies virus shown by sequencing to contain single point mutations in the G protein are nonpathogenic in adult mice (7). Variants of La Crosse virus (10) are reduced in their capacity to replicate in striated muscle, resulting in decreased viremia and spread to the CNS. In this case, the effect was systemic, because virus inoculated i.c. was fully neurovirulent.

Although we have no definitive evidence for such a mechanism in the MHV-4 system, the loss of ability of E2 to interact with cellular receptors is an attractive hypothesis to explain the loss of neurovirulence of the MHV-4 variants we selected. Further studies of the nature of the alterations in E2 and the tropism of the variants should enable us to assess the role of E2 in virus tropism and pathogenicity.

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