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Experimental demyelination induced by coronavirus JHM (MHV-4): molecular identification of a viral determinant of paralytic disease

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The molecular basis for demyelination induced by the neurotropic murine coronavirus JHM (JHMV or MHV4) is unknown. We have attempted to explore this issue by using neutralizing monoclonal antibodies specific for the major JHMV glycoprotein (E2) to select sets of neutralization resistant (NR) antigenic variant viruses. Monoclonal antibodies J.7.2 and J.2.2 bind to topographically distinct sites on E2. NR variants selected with J.7.2, like parental JHMV, predominantly cause a fatal encephalitis when given intracerebrally to mice, while J.2.2-selected NR variants cause a subacute disease characterized by paralysis and severe demyelination. We report here that consecutive selection with both J.2.2 and J.7.2 monoclonal antibodies results in NR variants which are markedly attenuated in both encephalitic potential and ability to induce demyelination. Analysis of the different variants suggests that the subregion of E2 bound by monoclonal antibody J.7.2 may be a critical viral determinant of paralysis and demyelination in this model system.

Key words: coronaviruses; demyelination; neurovirulence; antigenic variants; monoclonal antibodies; JHM (MHV-4).

Introduction

JHMV is a murine coronavirus strain which causes encephalitis and demyelination in mice. Along with other virus-induced and immunologically-mediated experimental diseases, JHMV infection has served as a model of human neurological illnesses.[1–5] It is reasonable to expect that study of these models may yield important insights and research strategies which may be relevant to human demyelinating diseases, including multiple sclerosis.[6–8] In this regard, a major goal has been to identify the molecular mechanisms responsible for experimental demyelination.

One problem in studying the mechanism of JHMV-induced demyelination has been the difficulty of separating myelin loss from the severe encephalitis which is also

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caused by the virus. In an effort to circumvent this problem, very restricted experimental conditions have been employed, such as studying all animals surviving one 50% lethal dose of JHMV.[4] Another approach has been to isolate mutants of JHMV which cause marked demyelination and minimal encephalitis.[9–11] Although these mutant viruses have yielded much useful and interesting information about JHMV pathogenesis, specific genetic lesions have not been identified in any of these mutants.

We and others have used monoclonal antibodies to select neutralization-resistant (NR) or antigenic variants of JHMV with altered biological properties.[12,13] Although these viruses have not yet been directly characterized by nucleic acid sequencing, several lines of evidence, including their selection frequency and antigenic properties, and also sequencing data on NR variants of other viruses,[14–19] suggest that the JHMV NR variants contain single point mutations. Thus, NR variants are ideal tools for studying viral pathogenesis, since the resulting disease may be linked to a discrete, definable genetic change in a single virus-encoded protein.[20]

Antigenic variants were initially selected with monoclonal antibodies specific for the major JHMV envelope glycoprotein, E2. E2 has several important biological activities, including virus attachment to target cells, induction of cell fusion, and the elicitation of neutralizing antibodies.[21–24] Thus, it was expected that viruses selected for putative mutations in E2 might have significant alterations in pathogenesis. As we have previously reported, JHMV NR mutants selected with monoclonal antibody J.2.2 were in fact found to have markedly reduced neurovirulence and to cause a subacute, paralytic demyelinating disease.[12] The J.2.2-selected variants had little or no tropism for neurons, and this feature may have accounted for their relative avirulence. Similar independently selected NR mutants of JHMV have been described by Dalziel *et al.*,[13] Leibowitz (personal communication), and Wege (personal communication), also indicating a central role for E2 in JHMV pathogenesis.

The availability of NR variants which predominantly cause a paralytic-demyelinating disease allowed us to study this phenomenon while minimizing the confouding factor of encephalitis. We report here on the sequential selection and pathogenic characterization of additional NR variants which are resistant to two anti-E2 monoclonal antibodies. These double-NR or multisite variants did not cause clinical paralysis, although mild to moderate residual demyelination was found histologically. These studies show for the first time that a putative single point mutation in one viral gene is correlated with the degree of paralysis and demyelination induced by the virus during central nervous system (CNS) infection.

Results

Selection of neutralization resistant variant viruses

Two JHMV-specific, neutralizing monoclonal antibodies, J.2.2 and J.7.2, were used to select JHMV variants resistant to neutralization. These monoclonal antibodies recognize separate sites on the major viral glycloprotein, E2.[12] Variant 2.2-V-1, originally selected from parental JHMV-DL for resistance to neutralization by monoclonal antibody J.2.2, was used to select additional, multisite variants based on the ability to resist simultaneous neutralization with both J.2.2 and J.7.2. Two plaque-purified isolates were studied in detail and designated 2.2/7.2-V-1 and 2.2/7.2-V-2. The biological properties of the consecutive or double variant viruses are summarized in Table 1.

Differential neutralization experiments (Table 2) showed that these double variants were resistant to neutralization with both selecting monoclonal antibodies. All viruses were sensitive to neutralization with mouse antisera to JHMV, and no virus was found

Table 1 Summary of pathogenic properties of JHM viruses

Virus	Type	Selection[a]	Disease[b]	Tropism[c] Region	Tropism[c] Cell type
JHMV-DL	parental	none	fatal encephalitis	GM, WM	both N and G
7.2-V-1	single NR variant	JHMV-DL×J.7.2	fatal encephalitis	GM, WM	both N and G
2.2-V-1	single NR variant	JHMV-DL×J.2.2	subacute paralysis	WM > GM	G only
2.2/7.2-V-1	double NR variant	2.2-V-1×J.2.2+J.7.2	none[d]	WM > GM	G, N rarely
2.2/7.2-V-2	double NR variant	2.2-V-1×J.2.2+J.7.2	none	WM > GM	G only

[a]Starting virus and monoclonal antibody used to select variants by escape from neutralization.
[b]Based on clinical observation of 6-week-old C57BL/6 mice for 19 days after i.c. inoculation of 1000 PFU of the indicated virus.
[c]Determined by immunohistochemical studies of brain and spinal cord, as described in the text. GM indicates gray matter; WM, white matter; N, neurons; and G, glial cells,. In addition, all viruses occasionally involved ependymal cells.
[d]Encephalitic signs, which were usually mild and transient, were seen in some mice infected with this virus.

Table 2 Comparative neutralizations of JHMV-DL and NR variant viruses

Neutralizing antibody	Antibody specificity[b]	Vitrus neutralization index[a] Viruses: JHMV-DL (parental)	2.2-V-1 (single NR variant)	2.2/7.2-V-1 (double NR variant)	2.2/7.2-V-2 (double NR variant)
J.2.2	E2—site B	4.1	0.8	0.0	0.0
J.7.2	E2—site A	4.1	1.5	0.0	0.0
J.2.2 + J.7.2	E2—sites B and A	4.1	4.8	0.0	0.2

[a]Neutralization index equals $\log_{10}$ PFU/ml of virus after neutralization with control antibody (anti-H-2I^p, reference 35) minus $\log_{10}$ PFU/ml of virus after neutralization with test antibody.[12] Thus, higher values of the index indicate increased neutralization.
[b]E2 is the peplomer or major JHMV glycoprotein. Competitive binding studies[12] have indicated that monoclonal antibodies J.2.2 and J.7.2 recognize two topographically distinct sites on E2, here designated site B and A respectively.

to exhibit a temperature-sensitive phenotype (data not shown). As we have previously reported,[12] when 2.2-V-1 was incubated with either J.2.2 or J.7.2 alone, there was little neutralization. However, when 2.2-V-1 was treated with both monoclonal antibodies simultaneously, there was marked neutralization. This finding implies that when used in combination monoclonal antibodies J.2.2 and J.7.2 neutralize 2.2-V-1 in a synergistic or supra-additive way.

Pathogenesis

The virulence of the viruses was compared by determining the 50% lethal dose (LD_{50}) in mice 7 days after intracerebral (i.c.) inoculation. The LD_{50} values for JHMV-DL, 2.2-V-1, 2.2/7.2-V-1 and 2.2/7.2-V-2 were approximately 4, 1×10^4, 1×10^4, and greater than 1×10^5 plaque forming units (PFU) respectively, indicating that the double variants retained the relatively avirulent characteristic of the single variant from which they were derived.

Clinical and histological CNS disease features were determined after i.c. inoculation of 1000 PFU of each virus. Mice given parental virus JHMV-DL developed an acute, fatal encephalitis. Clinical evaluation by a blinded observer of animals given variant

Table 3 Summary of clinical assessment of mice[a] inoculated with NR variant viruses

Virus	Mortality[b]	Encephalitic signs[c]	Paralysis[d]
2.2-V-1 (single NR variant)	0/10	0	7[f]
2.2/7.2-V-1 (double NR variant)	1/10	5[e]	0
2.2/7.2-V-2 (double NR variant)	0/10	0	0

[a]Each group consisted of six-week-old male C57BL/6 mice, given 1000 PFU i.c. of the indicated viruses and observed for 19 days p.i.
[b]Number of mice dead/number of mice inoculated.
[c]Number of mice showing signs of encephalitis during 19 days.
[d]Number of mice showing signs of paralysis during 19 days.
[e]Mild, transient signs in 4/5 mice. In one mouse the encephalitis was fatal.
[f]Severe paralysis was noted in 2/7 mice, mild paralysis in 5/7 mice.

viruses is shown in Table 3. The single NR variant 2.2-V-1 produced no encephalitic signs in mice; however, paralysis was evident in 7 of 10 animals. Paralysis was severe in 2 of the 7 mice. Significantly, neither double NR variant caused clinically evident paralysis, although the double variant 2.2/7.2-V-1 caused early, usually mild, signs of encephalitis in 5 of 10 mice. In only one instance did this progress to a fatal outcome.

In view of the clinical differences in mice given the paralytic single variant or the non-paralytic double variants, it was of interest to compare histological changes in the CNS of mice inoculated with these viruses. Acutely, at days 3–4-post inoculation (p.i), most mice infected with either the single or the double NR variants showed minimal mononuclear cell infiltration of gray and white matter. As noted, these lesions were subclinical, except for the transient early encephalitic signs seen in some mice given 2.2/7.2-V-1.

Subacutely, at days 19–21 p.i., there were little or no histologic abnormalities in the gray matter of the mice. Preliminary studies indicated that significant subacute pathological changes were limited to white matter regions. Accordingly, quantitative studies were done on coded sections of spinal cords obtained at 19 days p.i. (Fig. 1). Single variant 2.2-V-1 caused marked inflammation and myelin loss in 7 of 10 mice (Figs 1, 2(A)). These mice also showed paralysis clinically. By contrast, both double variant viruses caused only mild or moderate white matter lesions in the majority of mice examined (Figs 1, 2(B), 3(C)). Although these lesions were evident histologically, it is important to note that they were not of sufficient severity to cause observable paralysis.

As we have previously reported, NR variant 2.2-V-1 will cause a fatal, subacute encephalitis when given at high doses, such as 10^4 PFU i.c.[12] Therefore, additional experiments were performed to evaluate the behavior of the double NR variants when given at 10^4 to 10^5 PFU i.c. Under these conditions it was possible to produce a subacute, fatal encephalitis with variant 2.2/7.2-V-1, but not with NR variant 2.2/7.2-V-2. Although mice given high doses of either double NR variant continued to show foci of inflammation and myelin loss in the white matter, no mouse showed the extensive and severe demyelinating lesions characteristic of paralytic single variant 2.2-V-1 (Fig. 2(A)). Significantly, neither 2.2/7.2-V-1 nor 2.2/7.2-V-2 produced clinically evident paralysis at any dose. We conclude that the disease caused by the double NR variants (non-paralytic) is a different type than that caused by single NR variant 2.2-V-1 (paralytic); that is, the diminished demyelination caused by the double NR variants is not a quantitative effect which can be overcome by inoculating greater numbers of viruses.

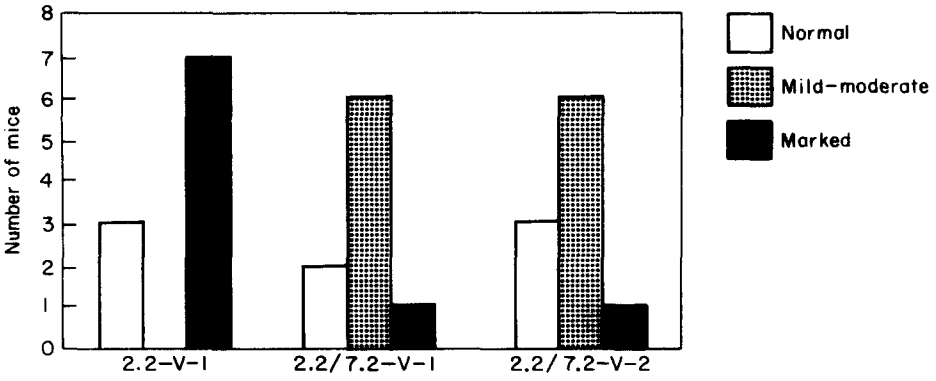

Fig. 1. Quantitative evaluation of the severity of white matter lesions in the spinal cords of mice 19 days after inoculation of 1000 PFU of the indicated viruses i.c. Histological grades were determined by a blinded observer, using the criteria given in the text.

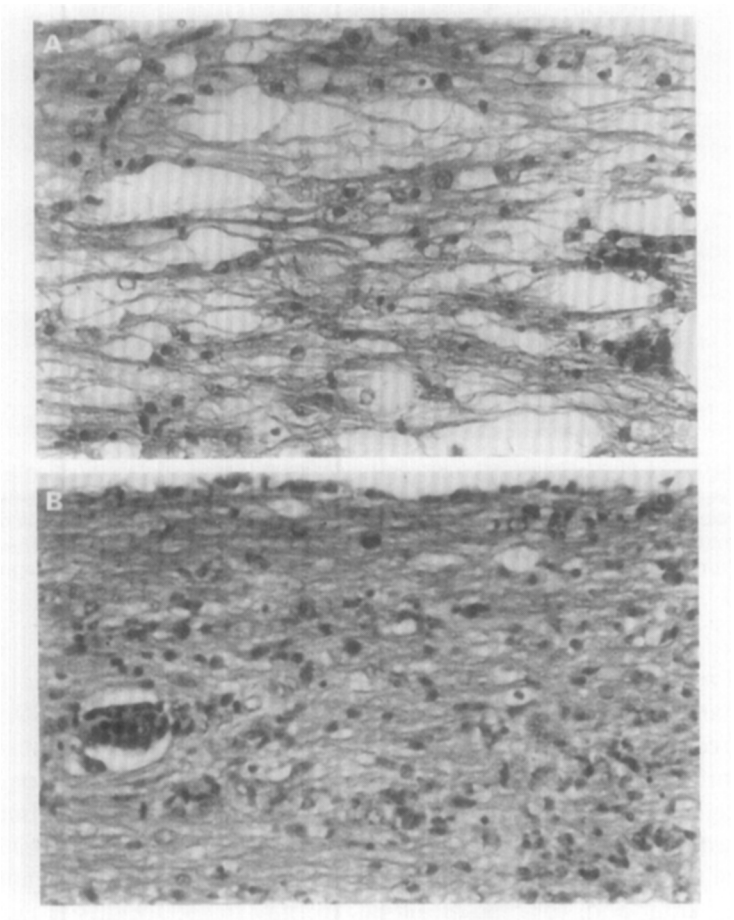

Fig. 2. Longitudinal sections of white matter in mouse spinal cord embedded in paraffin and stained with hematoxylin and eosin, 19 days p.i. with 1000 PFU of virus i.c. (A) Infection with single NR variant virus 2.2-V-1. Note marked inflammation and rarefaction. (B) Infection with double NR variant 2.2/7.2-V-1. Inflammation is less intense than in (A), and rarefaction is not evident. Magnification ×200.

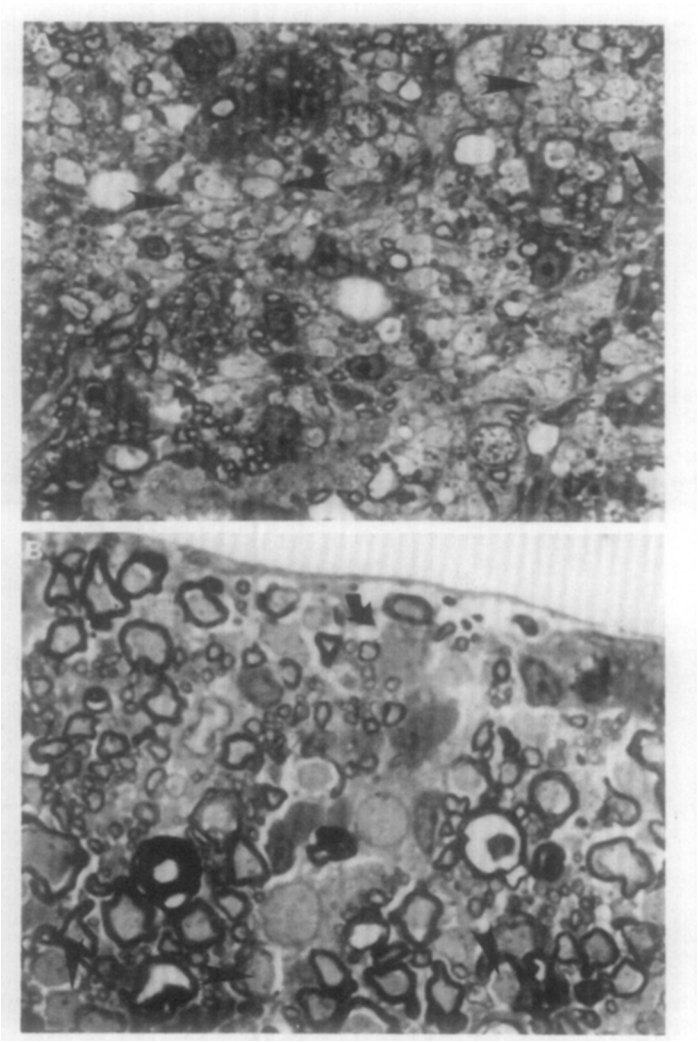

Fig. 3. Representative epon-embedded transverse sections of spinal cords stained with toluidine blue, from mice 19 days p.i. with 1000 PFU of virus i.c. (A) Infection with single NR variant virus 2.2-V-1. Note intense inflammation and naked, demyelinated axons (arrowheads). Magnification ×275. (B) Infection with double NR variant virus 2.2/7.2-V-1. Inflammation is most prominent subpially (arrow). Although there is some demyelination (arrowheads), this is much less extensive than with 2.2-V-1 (A). Magnification ×400.

In order to assess the initial target cells of the viruses, the immunohistochemical studies summarized in Table 1 were undertaken at 3–4 days after 1000 PFU of each variant was given i.c. In all cases, viral antigen distribution was multifocal, rather than uniform. Nevertheless, each virus showed a consistent predilection for certain CNS regions and cell types. For example, JHMV-DL antigen was found in both white and gray matter; both neurons and glial cells contained abundant viral protein (Fig. 4(A)). By contrast, mice inoculated with single NR variant 2.2-V-1 showed antigen in glial cells in white matter and, to a lesser extent, in gray matter (Fig. 4(B)); however, as we have reported previously,[12] this variant did not appear to infect neurons. Similarly, the double NR variant 2.2/7.2-V-1 also showed marked preference for white matter and glial cells (Fig. 4(C)); however, this variant occasionally infected neurons, although less frequently than the panencephalitic parental virus, JHMV-DL. Variant 2.2/7.2-V-1 usually involved neurons only when they were adjacent to heavily-

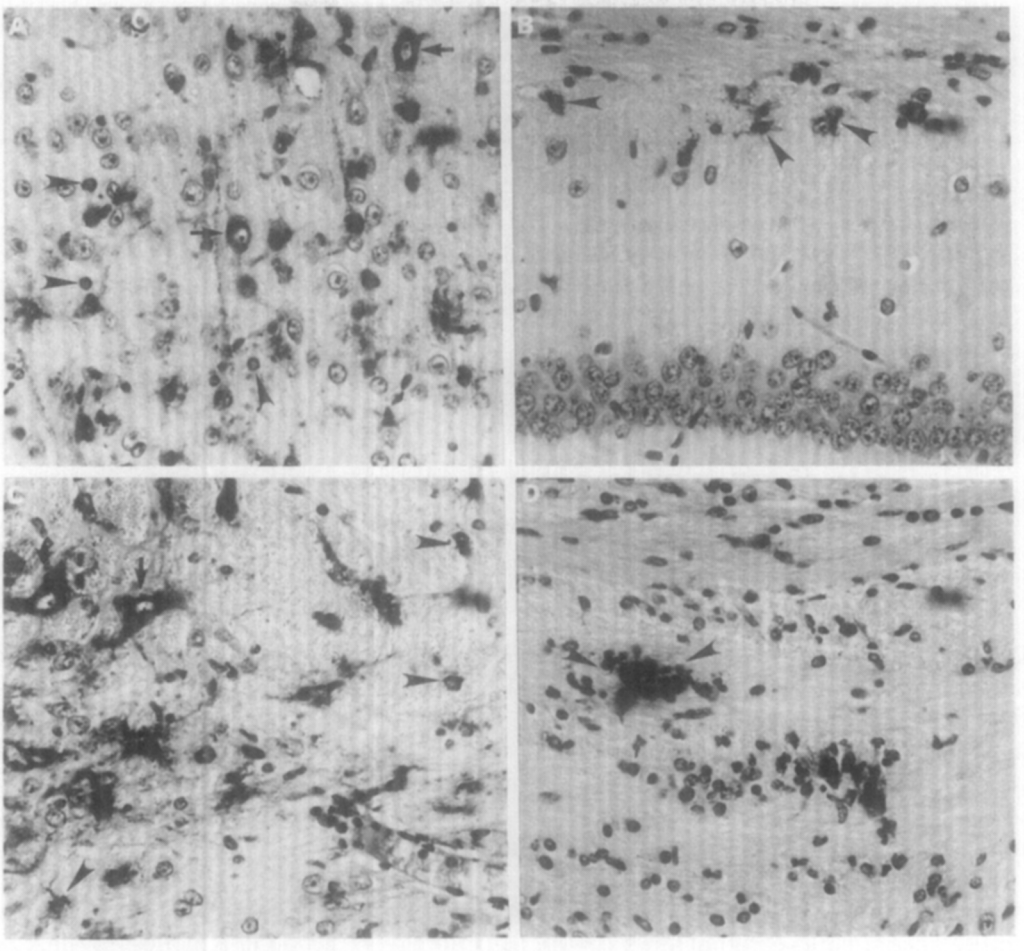

Fig. 4. Immunohistochemical demonstration of viral antigens in mouse brains 3 or 4 days p.i. with 1000 PFU of virus. (A) Infection with parental virus JHMV-DL, cerebral cortex. Antigen is present in both neurons (arrows) and adjacent glial cells (arrowheads). (B) Infection with single NR variant virus 2.2-V-1, corpus callosum and hippocampus. Although antigen is shown in glial cells (arrowheads), nearby neurons are antigen-negative. (C) Infection with double NR variant virus 2.2/7.2-V-1, midbrain. Glial cells are antigen positive (arrowheads), and two neurons also contain antigen (arrows). (D) Infection with double NR variant virus 2.2/7.2-V-2, corpus callosum. Note staining in glial cells (arrowheads). Magnification ×280.

involved white matter tracts; thus, unlike JHMV-DL, 2.2/7.2-V-1 did not affect neurons in the cerebral cortex. By contrast, mice infected with double NR variant 2.2/7.2-V-2 had very low levels of viral antigen, only demonstrable in white matter glial cells (Fig. 4(D)). Inconsistent infection of ependymal cells and meninges was seen with all viruses. Subacutely, at 19 days after infection with any of the three NR variants, low levels of viral antigen could occasionally be found only in the white matter.

Virus recovery experiments

In view of the differing disease patterns and lesions caused by the NR variant viruses, we sought to determine the extent to which each virus replicated and retained phenotypic stability *in vivo*. Figure 5 shows the kinetics of viral replication in the brains of mice inoculated i.c. with 1000 PFU of each virus. Maximal viral titers in brain were observed at day 3 with the NR variants and day 5 with the parental virus JHMV-

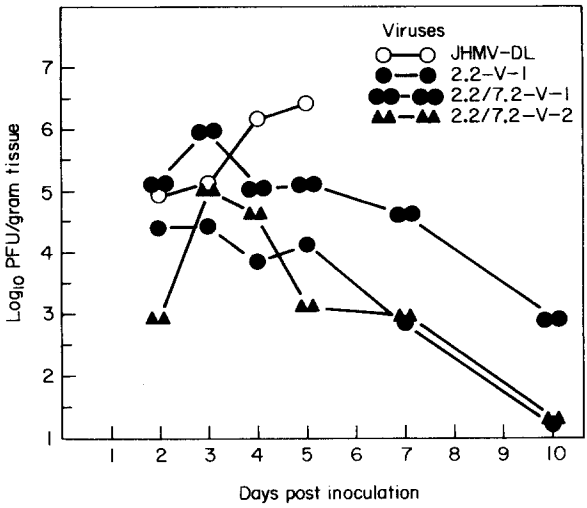

Fig. 5. Viral replication in the brains of mice injected i.c. with 1000 PFU of JHMV-DL (○), and NR variants 2.2-V-1 (●), 2.2/7.2-V-1 (●●), and 2.2/7.2-V-2 (▲▲). Each time point represents the mean titer from a group of three or six mice.

DL. The growth of the double variant 2.2/7.2-V-2 was virtually identical to that of the single variant 2.2-V-1. Variant 2.2/7.2-V-1, however, grew to relatively high titers, at times exceeding even those of JHMV-DL. The enhanced replication of 2.2/7.2-V-1, as well as the occasional involvement of neurons by this NR variant, may explain in part the mild encephalitic signs at 4–7 days p.i. shown by mice infected with this virus. The titers of virus recovered from spinal cords were approximately 1–2 $\log_{10}$ units lower than those of the respective brain homogenates.

We conclude that the relative avirulence of the variants was unlikely to be due to diminished replication, since avirulent variant 2.2/7.2-V-1 grew nearly as well as highly encephalitic parental virus JHMV-DL. Similarly, the diminished demyelination shown by the double variants also could not be attributed to reduced replication, since the viral titers in mice given the double variants were usually equal to or greater than those in mice infected with the single variant 2.2-V-1, which caused severe demyelination and clinically evident paralysis. All three variants showed phenotypic stability upon recovery at day 10; that is, they did not revert to the parental antigenic type, as judged by differential neutralizations performed as previously described.[12]

Discussion and conclusions

Monoclonal antibody-selected antigenic or NR variants of neurotropic viruses have proven useful in numerous studies of pathogenesis.[12,19,25–27] Where nucleic acid sequences have been determined, NR variant viruses have been shown to result from single point mutations. These strains thus represent powerful research tools, as discrete genetic changes can be correlated with pathogenicity. For example, RNA sequencing of rabies virus NR variants has shown that mutations affecting the amino acid in position 333 of the glycoprotein abolish neurovirulence; remarkably, substitutions at other positions, even as close as 330 and 336, do not affect lethality.[18] Similarly, Kaye and colleagues have shown that a single point mutation in the SI gene of reovirus type 3 alters CNS tropism and attenuates neurovirulence.[19]

We have extended the analysis of JHMV pathogenesis by sequentially selecting variants for resistance to neutralization by two monoclonal antibodies recognizing

separate antigenic sites on the major glycoprotein of JHMV, the E2 molecule. Two double NR variants, 2.2/7.2-V-1 and 2.2/7.2-V-2, were compared to the single variant (2.2-V-1) from which they were selected. While the double variants replicated relatively efficiently *in vivo*, they caused no clinically evident paralysis, in marked contrast to the paralysis readily produced by single variant 2.2-V-1. Histological studies showed that the severity of demyelination was substantially reduced in mice infected with the double NR variants. It is not known why the double NR variants, both selected by the same procedure, have minor pathogenic differences, for example, in relative tropism for neurons. In this regard, sequencing data on other NR variants has shown that a given monoclonal antibody may select several different mutations.[16] Alternatively, it is possible that one of the variants has an additional point mutation; however, in view of the selection frequency of JHM NR variants (approximately $10^{-4.5}$), we believe this explanation is unlikely.

Previous reports have pointed out that JHMV pathogenesis depends upon multiple host and viral factors.[5,28] Nevertheless, analysis of JHMV antigenic variants suggests that key properties of the virus E2 glycoprotein play important roles in determining disease outcome. For example, our prior study[12] showed that J.2.2-selected NR variants, such as 7.2-V-1, retain the lethality and tropism for neurons of parental JHMV-DL. By contrast, NR variants selected with monoclonal antibody J.2.2, such as 2.2-V-1, have reduced lethality and have little or no tropism for neurons. These findings imply that monoclonal antibody J.2.2 interacts with a site on the E2 molecule whose integrity, or parental configuration, is a critical determinant of acute fatal encephalitis. Dalziel *et al.*[13] and others (J. Leibowitz, H. Wege, personal communications), have reported similar findings.

The main finding of our present studies is that the double NR variants cause no paralysis clinically and reduced demyelination histologically. Presumably, a single point mutation in E2 distinguishes these variants from the single NR variant from which they were derived. These results imply that J.7.2 binds to a site on E2 which has a major influence on the process of demyelination. Studies with recombinant viruses[29] with crossovers in the E2 gene (Makino *et al.*, in press) suggest that the J.7.2 binding site may be near the carboxyl terminus of E2. Nevertheless, this subregion of E2 clearly cannot be the only viral determinant of demyelination, since the double NR mutants continue to cause low levels of subclinical myelin loss.

It is not known whether additional viral determinants of demyelination include regions of E2, near or distant from the J.7.2-binding site, or whether the residual demyelination depends upon additional JHMV gene products. Interestingly, comparative binding studies with available NR variants and monoclonal antibodies have demonstrated that the site recognized by J.7.2 is part of an overlapping complex in which at least three antigenically distinct subregions can be identified (Fleming, unpublished observations). Also, as has been shown with other viral proteins, the antigenic determinants of E2 may depend on complex intramolecular steric and conformational effects. These effects may explain the fact that variant 2.2-V-1 is resistant to monoclonal antibodies J.2.2 and J.7.2 when used separately but is consistently neutralized by these antibodies when used simultaneously. In other viral systems it has been demonstrated that the binding of one monoclonal antibody may enhance the binding of a second monoclonal antibody to a distant site on a viral protein.[30–33] The possibility that such synergism may underlie the action of J.2.2 and J.7.2 is currently under investigation.

In view of the complexity of the J.7.2 site, as well as the residual demyelination shown by the double NR variants, it may not be possible to define a single linear locus on E2 which is the necessary and sufficient cause of virus-induced demyelination.

Further studies, such as the selection of other variants, RNA sequencing of variants, determination of viral tropism for different types of glial cells, and the characterization of cellular receptors for JHMV, may clarify this issue. Finally, these studies of JHMV pathogenesis eventually must be considered in the context of host factors, including the immune system and the intrinsic resistance of different CNS cells to JHMV.

Materials and methods

Viruses, cells, animals, antibodies. The isolation and characterization of JHMV-DL, from which variant viruses were derived, as well as the derivation of single antigenic variant virus 2.2-V-1, have previously been described.[12] Viruses were propagated and plaque assayed on DBT or L2 cells.[34] Six-week-old male C57BL/6 mice (Jackson Laboratories, Bar Harbor, Maine), seronegative for murine coronaviruses, were used in all experiments 48–72 hours after receipt. Mice were inoculated i.c. with a 30 μl volume containing various numbers of PFU of virus as indicated in each experiment. Hyperimmune and monoclonal antibodies to JHMV were made in this laboratory.[22] Negative control antibody 7.16.17 (anti-H-21^{p}) was a gift from Dr Jeffrey Frelinger, University of North Carolina, Chapel Hill.[36] The temperature sensitivity of virus plaque formation was assayed as previously described.[12] Quantitation of infectious virus in brain and spinal cord samples was performed by assay of tissue homogenates on L2 cells as previously described.[212]

Selection of double antigenic variant viruses. Variant viruses were selected as previously described.[12] Briefly, variant virus 2.2-V-1 was incubated with an excess of neutralizing monoclonal antibodies J.2.2 and J.7.2. Surviving viruses were isolated and subjected to three cycles of plaque-purification. To neutralize any possible revertant viruses, incubation with the selecting monoclonal antibodies was performed prior to each plaque-purification. Before use, viruses were tested by differential neutralization[12] with the selecting monoclonal antibodies and negative control antibody, 7.16.17; if the titers of the two assays were within 1 $\log_{10}$ unit of each other, the virus was considered to be resistant to neutralization. Viruses failing to meet this criterion were considered revertants and were discarded.

Clinical observations. The clinical status of infected mice was judged by a modification of the criteria of Brown *et al.*[36] Animals were considered normal if they appeared alert and could turn over within 2 seconds after being placed on their backs. Mice that had a waddling gait and inability to rapidly turn over were scored as having mild paralysis. Mice with frank hindleg immobility were scored as having severe paralysis. Animals with hyperirritability or myoclonus were judged to have mild encephalitic signs; seizures, persistent turning, or static, hunched postures were considered to be signs of severe encephalitis. Mice were scored by a single examiner after the cages had been coded with randomized numbers.

Histology. Brain and spinal cords were embedded in paraffin or epon and stained as previously described.[12] The severity of histologic lesions was assessed by grading randomized, coded microscopic sections. Gray matter involvement was considered severe if lesions were extensive and there was marked inflammatory cell infiltration and neuronophagia; lesser degrees of involvement were scored moderate or mild. White matter involvement was considered severe if there was intense, widespread inflammatory infiltrates with rarefaction; white matter involvement with less inflammation and no tissue rarefaction was scored as moderate or mild, depending on the extent of lesions. Demyelination was evaluated in paraffin-embedded sections stained with luxol fast blue or with epon-embedded sections stained with toluidine blue. For immunohistochemistry, sections of paraffin-embedded tissue were stained by an avidin-biotin immunoperoxidase procedure (Vectastain, Vector Laboratories, Burlingame, California), using monoclonal antibodies to the JHMV N protein[23] as primary antibodies.

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