PATHOGENIC CHARACTERISTICS OF NEUTRALIZATION-RESISTANT VARIANTS OF JHM CORONAVIRUS (MHV-4)

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Introduction

One of the most interesting aspects of murine coronaviruses is their ability to cause model, experimental neurological diseases in animals (1-10). In this situation, disease obviously depends on complex interactions between virus, host, and environment. The initial focus of our group has been to consider the specifically viral determinants of disease. We have adopted this approach because the virus is the least complex element of this biological system. Hopefully, the elucidation of viral components which play critical roles during disease will provide a basis for more general explanations of pathogenesis, including consideration of the host and environment.

The viral determinants of disease are often studied by producing mutant viruses. Mutants can be used to infect animals, and the diseases resulting from viruses with different mutations may be compared. Mutant analysis may lead to information about the role of specific viral proteins in pathogenesis. In this regard, it has been shown that a very useful type of mutant virus for these studies may be selected by neutralizing monoclonal antibodies. A variety of equivalent terms have been used to describe these viruses, including antigenic variants, escape mutants, monoclonal antibody resistant mutants, and neutralization-resistant (NR) variants. To produce NR variants, a population of parental or wild type virus is incubated with an excess of neutralizing monoclonal antibody. A fraction of the original population survives neutralization by virtue of pre-existing mutations which allow some viruses to escape recognition by the antibody. Available sequencing data on NR variants indicates that they arise by single point mutations (11-16). NR variants have been successfully employed to study a variety of neurotrophic viruses (15-19).

We and others (Dalziel *et al.* (20); Wege *et al.*, and Leibowitz *et al.*, in this volume) have used neutralizing monoclonal antibodies to select NR variants of JHM murine coronavirus (JHMV or MHV-4). The frequency with which NR variants occur in parental JHMV populations has been found to be about $10^{-4.5}$, which is consistent with the experience with other RNA viruses (21) and probably reflects the high mutability of RNA viruses in general (22). Since the genome of JHMV is approximately 18 kilobases (23), this selection frequency for NR variants implies that on average each variant has only one mutation *viz* a *viz* the parental genotype. Furthermore, the finding that a given monoclonal antibody typically selects variants which cause the same type of disease (see below)

implies that random, "second mutations", if they do occur, are usually of little pathogenic importance.

This chapter summarizes our experience with NR variants which were selected with monoclonal antibodies to the JHMV E2, or major glycoprotein. We first describe the selection and characterization of single NR variants, that is, variants selected from parental JHMV in one step by one monoclonal antibody. These single NR variants are likely to have single point mutations in the E2 gene. Secondly, we discuss double NR variants, that is, variants selected by incubating single NR variants with a second monoclonal antibody. These double or multisite variants are presumed to have two point mutations. Some of this research has been presented elsewhere (24,25).

Materials and Methods

Viruses, antibodies, and cells

A large plaque strain of JHMV, JHMV-DL (26), was used as a starting or parental virus. NR variants were selected from JHMV-DL as previously described (24,25). Briefly, approximately 10^6 plaque forming units (PFU) of virus were incubated with an excess of neutralizing anti-E2 monoclonal antibody. Variants resistant to neutralization were grown on DBT or L2 cells (27) and subject to three cycles of plaque-purification in the presence of monoclonal antibody. NR variants were selected with two monoclonal antibodies, J.7.2 and J.2.2; as determined by competitive binding studies, these antibodies recognize topographically distinct sites on the E2 molecule (24). Single NR variants selected with monoclonal J.7.2 were represented by variant 7.2-V-1, while those selected with J.2.2 were represented by variant 2.2-V-1. Two double NR variants were selected by incubating variant 2.2-V-1 with both monoclonal antibody J.7.2 and J.2.2, and these multisite variants were designated 2.2/7.2-V-1 and 2.2/7.2-V-2. Prior to use, all viruses were assessed by differential neutralizations (24), to assure that they had not reverted to the parental phenotype. The binding of antibodies to different viruses was measured by radioimmunoassay (28) or enzyme-linked immunoabsorbent assay (ELISA) (24), as previously described.

Pathogenesis

Six week old C57BL/6 male mice were used in all experiments. Representative mice were tested on arrival by ELISA to assure that they did not have reactivity to mouse hepatitis viruses. Mice were inoculated intracerebrally (i.c.) with virus 48-72 h. after arrival. Viral titers in mice were determined by plating tissue homogenates on L2 cells (24). Tissues were fixed with formaldehyde or Clark's fixative and embedded in paraffin. Sections were stained with hematoxylin and eosin for routine histology, luxol fast blue for myelin, Bodian silver for axons, and phosphotungstic acid for glial fibers. Immunohistochemistry, using the avidin-biotin technique and monoclonal antibodies to JHMV N protein to demonstrate viral antigen, was performed as described (25).

Results

Single NR Variants

Monoclonal antibodies J.7.2 and J.2.2 each were used to select a series of NR variants. Four NR variants selected with J.7.2 were found to have general pathogenic features which were unchanged from parental virus JHMV-DL; that is, when given to mice at a dose of 10^3 PFU i.c., they all caused an acute fatal encephalitis. By contrast, four NR variants selected with J.2.2 were non-lethal at this dose, and the majority of the animals developed a subacute paralytic disease. In view of the generality of our finding that the two monoclonal antibodies consistently selected the same sort of NR variant (J.7.2, encephalitic;

J.2.2, paralytic), two representative variants, 7.2-V-1 and 2.2-V-1, were subsequently studied in detail (24).

The antigenic properties of the NR variant viruses were assessed by comparing their neutralization by different antibodies (Table I). Mouse antisera to JHMDL efficiently neutralized JHMDL, 7.2-V-1, and 2.2-V-1. Both NR variants were resistant to their selecting monoclonal antibodies, that is, 7.2-V-1 to J.7.2 and 2.2-V-1 to J.2.2. Variant 7.2-V-1 was efficiently neutralized by J.2.2; however, variant 2.2-V-1 unexpectedly was found to be partially resistant to J.7.2, the antibody not used in its selection. Both NR variants had full ability to form plaques at 32°, 37°, and 39°.

TABLE I. Neutralization of Different Viruses by Polyvalent and Monoclonal Antibodies¹

Virus	Type (reference)	Antibodies ²			
		Anti-JHMDL	J.2.2	J.7.2	J.2.2 and J.7.2
JHMDL	Large plaque (26)	+	+	+	+
7.2-V-1	Single NR variant selected by J.7.2 (24)	+	+	-	+
2.2-V-1	Single NR variant selected by J.2.2 (24)	+	-	±	+
2.2/7.2-V-1	Double NR variant selected by J.2.2 and J.7.2 (25)	+	-	-	-
2.2/7.2-V-2	Double NR variant selected by J.2.2 and J.7.2 (25)	+	-	-	-

1. Neutralization was determined by incubation with antibody and subsequent plaque assay on DBT cells, as previously described (24,25). Complete neutralization is indicated by a "+" sign, and complete resistance to neutralization is indicated by a "-" sign. In one instance partial neutralization was found (24), as indicated by a "±" sign.

2. Anti-JHMDL serum was obtained from hyperimmunized mice and used at a 1:100 dilution. The indicated monoclonal antibodies were used at a 1:10 dilution of spent hybridoma supernate.

NR variant 7.2-V-1 presumably contains a mutation affecting the integrity of a region which we have designated "A" and which is recognized by monoclonal antibody J.7.2. The 50% lethal dose (LD₅₀) for 7.2-V-1 was found to be 25 PFU, which is not significantly different from the LD₅₀ of parental JHMDL, i.e., 4 PFU (24). Mice given 7.2-V-1 developed signs of encephalitis (myoclonus, seizures, obtundation) by day 3 or 4 post-inoculation (p.i.) and were dead by day 7 p.i. Histologic examination of the central nervous system (CNS) of these mice revealed marked small cell infiltration of both gray and white matter, and immunohistochemical studies demonstrated viral antigen in all major cell types, including neurons (figure 1A) (24). Thus alterations at site A on E2 do not seem to diminish virulence or lethality.

NR variant 2.2-V-1 is representative of viruses with a mutation affecting the integrity of a second region of E2, recognized by monoclonal antibody J.2.2 and designated "B". The LD₅₀ of variant 2.2-V-1 was found to be approximately 10⁴ PFU (24). Approximately 70% of mice given 10³ PFU of 2.2-V-1 developed hindleg paralysis, which was first evident by day 8 or 9 p.i. and was maximal at about day 14 p.i. Histologic examination revealed mild inflammatory changes in both CNS gray and white matter acutely at day 4 p.i. Immunohistochemical studies revealed viral antigen in glial cells; unequivocal staining of neurons was

not observed (figure 1B). By day 19 p.i. significant lesions were limited to the white matter, where severe primary demyelination was observed (24). Demyelination was also noted at later stages of infection; these lesions appeared active at 30 days p.i. and inactive 12 months p.i. (24).

The replication of NR variants was investigated *in vivo*, since a failure of growth might account for the changes observed in pathogenesis. Maximal viral titers were noted at 3-4 days p.i. and were $10^{6.7}$, $10^{5.8}$, and $10^{5.3}$ PFU/g brain for JHMV-DL, 7.2-V-1, and 2.2-V-1 (24), respectively. These data thus indicate that the NR variants replicate slightly less efficiently than parental virus; however, when the variants are compared to each other, the relatively small difference in growth observed would not appear to explain the marked difference in virulence between 7.2-V-1 and 2.2-V-1. Differential neutralizations revealed that viruses recovered from murine brain at days 5-6 p.i. retained the phenotype of the input variant administered (data not shown).

Double RN Variants

The availability of JHMV NR variant 2.2-V-1, which was relatively non-lethal and caused severe paralysis in mice, allowed us to investigate viral-induced demyelination while minimizing the confounding factor of encephalitis, which is prominent in parental JHMV-DL infection (26). This variant was used in further selections employing both monoclonal antibody J.7.2 and monoclonal antibody J.2.2. The resulting double or multisite NR variants were designated 2.2/7.2-V-1 and 2.2/7.2-V-2. Both double variants were completely resistant to neutralization with J.7.2 and J.2.2 singly or in combination (Table I). Thus, the double variants are presumed to have mutations affecting the integrity or parental configurations of both site A and site B on E2.

Both double variants 2.2/7.2-V-1 and 2.2/7.2-V-2 were relatively avirulent when mice were inoculated i.c.; the LD_{50's} were approximately 10^4 PFU and greater than 10^5 PFU respectively. In order to assess disease patterns caused by the double variants, groups of mice were given 10^3 PFU of each virus i.c. Clinical status and histologic evaluation were performed by a blinded observer. Both double variants failed to cause clinically evident paralysis, although the single variant from which they were derived (2.2-V-1) induced paralysis in 70% of mice. Transient encephalitic signs were seen in approximately one-half of the mice given 2.2/7.2-V-1, but in only 1 of 10 mice did this progress to death (25). Histologic examination at 19 days p.i. showed that the lesions caused by the double NR variants were principally in the CNS white matter, and quantitative evaluation revealed that the intensity of inflammation and degree of demyelination induced by the double variants were substantially less than that caused by single NR variant 2.2-V-1 (25). Immunohistochemical studies showed that 2.2/7.2-V-1 predominantly infected white matter glial cells, probably oligodendroglia; occasionally neurons were involved to a lesser degree (figure 1C). NR variant 2.2/7.2-V-2 antigen was only present in low levels and in white matter glial cells (figure 1D).

Studies of replication *in vivo* indicated that the growth of double variant 2.2/7.2-V-2 was virtually identical to the highly paralytic single NR variant 2.2-V-1 (25). Thus, a failure of replication in the CNS cannot account for the lack of paralysis caused by the double variant. In fact, the other double NR variant, 2.2/7.2-V-1, achieved a maximum titer approximately $10^{5.5}$ PFU/g higher than 2.2-V-1 or 2.2/7.2-V-1. The robust growth of 2.2/7.2-V-1 may explain the propensity of this variant to cause mild encephalitic signs. Both double variants were phenotypically stable upon recovery at day 10 p.i., that is, differential neutralizations did not indicate reversion to the antigenic profile of parental JHMV-DL or single NR variant 2.2-V-1 (data not shown).

Figure 1: Immunohistochemical demonstration of JHMV antigen in mice acutely infected with JHMV NR variants. Brains or spinal cords were obtained from mice sacrificed 4 days p.i. with 10^5 PFU of the indicated viruses. Viral antigen was demonstrated by means of monoclonal antibodies to the N protein as indicated in the text. (A) Single NR Variant 7.2-V-1. Neurons (arrows) and glial cells (arrowheads) contain viral antigen. (B) Single NR Variant 2.2-V-1. Viral antigen in glial cells only (arrowheads). Note normal hippocampal neurons (star). (C) Double NR Variant 2.2/7.2-V-1. Viral antigen is shown in glial cells (arrowheads) predominantly, although less frequently staining of neurons (arrows) is also present. (D) Double NR Variant 2.2/7.2-V-2. Low levels of viral antigen demonstrable in glial cells (arrowhead) only. Hippocampal neurons are normal (star). Original magnification x100, insets x 160.

Discussion

It is probable that the pathogenesis of JHMV, like that of other viruses, is complex and will be shown to depend on many viral factors. In the well-studied reovirus and influenza virus infections, for examples, it has been demonstrated that several viral proteins play key roles at different stages in the disease process; stated differently, pathogenesis is multigenic (29). With regard to MHVs, our previous survey of the antigenic properties of different strains found correlations between pathogenesis and alterations of the viral N protein (28). Also, in all probability, mutations in viral gene products such as the leader sequence (30) or polymerase (31) will heavily influence the efficiency of JHMV replication and, therefore, pathogenesis. Nevertheless, most studies of this issue to date have focused the E2 protein, and our studies of NR variants also indicate a central role for E2 in control of disease outcome.

Figure 2: Schematic representation of a topographical map of antigenic sites we have identified on the E2 molecule (top right) and the derivation and characteristics of NR variants (bottom left). Different virus strains are shown within boxes. The type of disease caused by each virus is shown within parentheses; E⁺ indicates acute fatal encephalitis, and P⁺ indicates subacute paralytic-demyelinating disease. The negative sign indicates the absence of these disease types, and a question mark indicates that disease cannot be assessed, as when acute fatal encephalitis prevents evaluation of subacute paralytic-demyelinating disease. The immunoselection of NR variants is shown by arrows, and the selecting monoclonal antibodies are shown above each arrow.

Our current understanding of topographical regions of E2 is schematically shown at the top right of figure 2. In competition assays with our panel of neutralizing anti-E2 monoclonal antibodies, two topographically distinct sites on E2 have been identified (24). The site recognized by monoclonal antibody J.7.2 has been arbitrarily designated "A" and the site recognized by monoclonal antibody J.2.2 has been designated "B". Preliminary evidence indicates that site A may be homologous to site A of Wege *et al* (32; Wege, personal communication) which is one of two antigenic regions they have identified which binds neutralizing monoclonal antibodies. Talbot *et al.* (33) have independently identified three topographically distinct sites on E2 which bind neutralizing monoclonal antibodies. At present, Talbot and colleagues are comparing available panels of anti-E2 monoclonal antibodies in order to clarify the relationships among antigenic regions identified by different groups. Data from recombinant MHV's with cross-overs in the E2 gene (gene C) indicate that sequences within 1.5 KB of the 3'-end of this gene code for essential determinants in the antigenic regions we have designated A and B (Makino *et al.*, submitted). Site B appears to be dependent upon the most 3' regions of the gene. In figure 2, the letter C is used to indicate additional sites on E2 which we and others have noted (32;33; Fleming, unpublished observations). These findings, based upon variant and recombinant JHMV strains, need to be examined in light of the recently-published E2 nucleotide sequence for parental JHMV (34).

The bottom left of figure 2 diagrammatically demonstrates our experience with NR variants of JHMV. The parental or starting virus was JHMV-DL, an isolate of JHMV which causes large plaques and is highly encephalitic. Strictly speaking, the ability to cause subacute paralytic disease is unknown since in the experiments above all mice given JHMV-DL died acutely of encephalitis. These characteristics are thus represented as the ($E^+ P^-$) phenotype. When monoclonal antibody J.7.2 was used to select NR variants from parental JHMV-DL, the isolates selected, as represented by 7.2-V-1, remained highly encephalitic. Again, since all mice given 7.2-V-1 died of acute encephalitis, the ability to cause subacute or chronic demyelination could not be assessed. In marked contrast, the selection of NR variants such as 2.2-V-1 by monoclonal antibody J.2.2 resulted in viruses which were highly attenuated in their ability to cause fatal encephalitis. Under these circumstances, the majority of mice developed paralysis subacutely. Histologic examination of these mice revealed intense primary demyelination. Similar NR variants have been reported by Dalziel *et al.* (20), Wege *et al* (in this volume), and Leibowitz *et al.* (in this volume).

In conclusion, selection of variants with monoclonal antibody J.2.2 results in viruses with attenuated neurovirulence, while selection with J.7.2 does not. Thus the normal or parental configuration of the site we have designated B on E2 appears to be necessary for acute, fatal encephalitis. The effect of alterations in E2 site A on demyelination cannot be determined directly with the single NR variants, since in any virus with site B intact encephalitis predominates clinically, and mice do not survive into the time period in which paralysis and marked demyelination are usually manifest. On the other hand, using double or multisite NR variants, it was possible to study the effects of mutations affecting the site on E2 we have designated A. Single NR variant 2.2-V-1 was used as a starting virus. This variant with a putative mutation in E2 site B was attenuated in neurovirulence but caused marked paralysis and demyelination. By adding monoclonal antibody J.7.2 (recognizing E2 site A) to the selection procedure, it was possible to derive double variants which appeared to have antigenic alterations at both E2 sites A and B. These NR variants did not cause observable paralysis, although low levels of demyelination was evident histologically. Since neutralization of 2.2-V-1 with the ($E^- P^+$) phenotype by monoclonal antibody J.7.2 selected variants such as 2.2/7.2-V-1 and 2.2/7.2-V-2 with the ($E^- P^-$) phenotype, we have concluded that site A is a region of E2 whose configuration is a major determinant of paralytic disease.

The mechanisms which account for the pathogenic differences among NR variants is unknown. Since E2 is considered to function as the viral attachment protein (23), it is quite possible that alterations in E2 may alter JHMV tropism for critical CNS cells, such as neurons or oligodendroglia. Nevertheless, other possible roles for E2 must be considered, such as influencing viral replication at stages after attachment. Alternatively, alterations in E2 may cause important changes in the way the immune system interacts with JHMV.

Conclusions

1. Variant JHM viruses selected for resistance to neutralization (NR) to different monoclonal antibodies cause different neurological diseases in mice.
2. These NR-variants in all probability contain point mutations.
3. Alterations in the E2 viral glycoprotein region we have called B (binding monoclonal antibody J.2.2) have a major influence on the lethality and encephalitic potential of JHM virus.
4. Alterations in the E2 viral glycoprotein region we have called A (binding monoclonal antibody J.7.2) have a major influence on the paralysis and demyelination caused by the virus.
5. To date the effects associated alterations in E2 sites A and B are not absolute. Interactions with other E2 sites, other JHM virus gene products, and the host must be considered in pathogenesis.

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