A Neutralization Site of DA Strain of Theiler's Murine Encephalomyelitis Virus Important for Disease Phenotype

SHIGERU SATO,*1 LIANG ZHANG,*1 JONG KIM,* JOHN JAKOB,* ROBERT A. GRANT,† ROBERT WOLLMANN,‡ and RAYMOND P. ROOS*2

Department of *Neurology and †Pathology, University of Chicago Medical Center, Chicago, Illinois, 60637; and ‡Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115

Received May 15, 1996; returned to author for revision June 25, 1996; accepted October 10, 1996

DA strain of TMEV induces a chronic, persistent, demyelinating disease in SJL/J weanling mice, while inoculation with GDVII strain of TMEV induces an acute, lethal neurovirulent disease. We show that three amino acids in the DA EF loop—DAVP2 141 Lys, 143 Gly, and 173 Thr—are part of a neutralization site of DA monoclonal antibody (mAb), DAMAb1. DA virus with a mutation of VP2 143 from Gly to Asp, like wild-type virus, persists 6 weeks postinfection (PI) and produces white matter disease. DA virus with a mutation of VP2 141 from Lys to Asn persists but does not induce significant white matter disease. DA virus with a mutation of DA VP2 173 from Thr to Phe fails to persist or to induce significant white matter disease. The diversity and complexity of the mutant virus-induced disease phenotype presumably reflects the varied effects of the mutated amino acid residues on the three-dimensional structure of the viral capsid. The localization of DA VP2 141 and VP2 173 near the putative receptor binding region of the virus suggest that a disruption of interactions between the virus and its receptor is important in the late demyelinating disease and for virus neutralization.

INTRODUCTION

Theiler's murine encephalomyelitis virus (TMEV) designates a group of closely related strains of a picornavirus that cause enteric and central nervous system (CNS) disease in weanling mice (reviewed in Roos and Casteel, 1992). DA strain and other members of the TO subgroup cause a biphasic disease, initially infecting neurons and then inducing a persistent, demyelinating infection of the CNS (Lipton, 1975; Lipton and Dal Canto, 1978). The latter disease serves as an experimental model of multiple sclerosis (MS). In contrast, inoculation of weaning mice with GDVII strain and other members of the GDVII subgroup leads to an acute, fatal polioencephalomyelitis (Roos and Casteel, 1992).

To identify viral genes responsible for the different phenotypes, investigators have generated full-length infectious cDNA clones of both DA and GDVII and then constructed chimeric cDNA clones with parts of DA and GDVII exchanged for one another. The resultant recombinant viruses have been inoculated into animals to elucidate the molecular determinants of neurovirulence, demyelinating activity, and persistence (Pritchard et al., 1993; Tangy et al., 1991; Zhang et al., 1993). These studies have demonstrated, for example, that the GDVII cap-

1 S. Sato and L. Zhang contributed equally to this study.
2 To whom correspondence and reprint requests should be addressed. Fax: (312) 702-7775. E-mail: roos@drugsbsd.uchicago.edu.
FIG. 1. Wild-type and mutant TMEV and their phenotypes. Segments derived from the pDAFL3 parental infectious clone (Roos et al., 1989a) are shown as open bars; segments derived from the pTMDA parental infectious clone (McAllister et al., 1989) are shown as shaded bars; segments derived from the pGDVII infectious clone (Fu et al., 1990) are shown as black bars. The TMEV genome organization is shown at the bottom: the 5' UTR and 3' UTR represent the 5' and 3' untranslated regions, respectively; L encodes the leader protein; P1 encodes the precursor for capsid proteins 1A (VP4), 1B (VP2), 1C (VP3), and 1D (VP1); P2 and P3 encode the precursors for nonstructural proteins. The circle shows the presence of a Lys (open) or Asn (shaded) as VP2 141 for the topmost 5 viruses; the bottom five viruses all have a Lys as VP2 141 (since they have a DAFL3 backbone), but have a mutant residue elsewhere in VP2 which is specified and shown as a speckled circle. The sensitivity (+) or resistance (−) of the particular virus to neutralization by DAmAb1 is noted; also see Table 1 and the Results section. The ability of these viruses to induce white matter pathology (meningitis, inflammation, and demyelination) is scored from − to +++ as described under Materials and Methods and derived from the mean value (see Table 4). The approximate white matter pathology grades of TMDA, GD1B-2A/DAFL3, and TMR4 viruses are taken from a previous publication (Jarousse et al., 1994). ND = not determined.

et al., 1994); it remained unclear, however, whether the presence of Asn as VP2 141 affected the ability of the parental DA strain (as well as the recombinant TMR4 virus) to demyelinate and persist. One of the goals of the present study was to clarify the role of VP2 141 and surrounding amino acids in the TO subgroup disease phenotype.

The crystallographic three-dimensional structures of two members of the TO subgroup, DA and BeAn, have been solved (Grant et al., 1992; Luo et al., 1992). The

TABLE 1

<table>
<thead>
<tr>
<th>Dilution of DAmAb1</th>
<th>DAFL3</th>
<th>DA-VP2-141 (Asn)</th>
<th>DA-VP2-143 (Asp)</th>
<th>DA-VP2-148 (Ala)</th>
<th>DA-VP2-162 (Ser)</th>
<th>DA-VP2-173 (Phe)</th>
</tr>
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<tbody>
<tr>
<td>10^{-10}</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>10^{-12}</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>10^{-14}</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>10^{-16}</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
</tbody>
</table>

* Neutralization assays were performed by using 100 PFU of either wild-type DAFL3 virus or mutant virus with various dilutions of DAmAb1. The result was scored as positive if there was a 50% or greater reduction in the number of PFUs.
three major capsid proteins VP1, VP2, and VP3, have an eight-stranded antiparallel \( \beta \)-barrel core structure, as is true for other picornaviruses. Unique loop structures that connect the \( \beta \)-barrels extend outward into space from the capsid surface and constitute potential antigenic sites. DA VP2 141 is located in the EF loop of VP2 (which spans VP2 amino acid 136 to 181) and arises from the surface of the virion, suggesting a role for this region in virus neutralization — especially since a neutralization site in the "EF loop" has been identified in the case of Mengo virus, a cardiovirus related to TMEV (Boege et al., 1991). In the present study, we demonstrate that the EF loop of VP2 contains epitopes for a previously described neutralizing monoclonal antibody, DAmAb1 (Nitayaphan et al., 1985), and is important for the late demyelinating white matter disease.

### MATERIALS AND METHODS

#### Cells

BHK-21 cells (baby hamster kidney cells) were obtained from American Type Culture Collection and used for transfection, plaque assays, and the growth of stock virus.

#### Construction of mutant plasmids

The template for the construction of mutants was the full-length, infectious cDNA clone of DA strain known as pDAFL3 (Roos et al., 1989a). pDAFL3 was made into a full-length, single stranded, uracil-containing template according to methods provided in a Muta-gene kit (Bio-Rad, Hercules, CA). The mutant plasmids that were constructed (and the oligonucleotide primer used for the mutagenesis reaction (Operon, Alameda, CA) shown 5' - 3' with nucleotide mismatches underlined) are: pDA-VP2-141 (Asn) (CCATGTCACTTTTGGGCC), with a change in amino acid VP2 141 from Lys to Asn; pDA-VP2-148 (Asp) (GGCTTACATGTCAGTTTGTCC), with a change in amino acid VP2 148 from Thr to Asp; pDA-VP2-143 (Asp) (AAAGGGTCAGCGGCTCATG), with a change in amino acid VP2 143 from Gly to Asp; pDA-VP2-162 (Ser) (AGTGGGCGACTTTGCGGGCC), with a change in amino acid VP2 162 from Gly to Ser; and pDA-VP2-173 (Phe) (GCGAAGAAACCGGCGCTATCGTA), with a change in amino acid VP2 173 from Thr to Phe. Each primer was annealed in a separate reaction to produce a different mutant plasmid. Double-stranded cDNAs were then generated from these single stranded/annealed primers and used to transform Supercompetent Escherichia coli cells (Stratagene, La Jolla, CA). The plasmids were sequenced using the dsDNA sequencing kit ( Gibco BRL, Gaithersburg, MD) to confirm the presence of the changes.

Preparation of DAmAb1 neutralization escape mutant viruses

TMEV mAbs were prepared and characterized as previously described (Nitayaphan et al., 1985). A wild-type DA virus stock was plaque-purified three times before use in the generation of mutant viruses resistant to DAmAb1. Mutants were isolated by growing the virus with mAb on BHK-21 cells for 4–7 passages and then performing a plaque assay in which L cells were infected with serial 10-fold dilution of the resistant virus and then

### TABLE 2

<table>
<thead>
<tr>
<th>VP2 Residue</th>
<th>DAmAb1 escape mutant virus Nos. 1-4</th>
<th>DAmAb1 escape mutant virus No. 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>143 Gly</td>
<td>Asp</td>
<td>Gly</td>
</tr>
<tr>
<td>148 Thr</td>
<td>Thr/Ala</td>
<td>Ala</td>
</tr>
<tr>
<td>162 Gly</td>
<td>Gly/Ser</td>
<td>Ser</td>
</tr>
<tr>
<td>173 Thr</td>
<td>Thr</td>
<td>Phe</td>
</tr>
</tbody>
</table>

* DAFL3 virus was derived from transfection of the pDAFL3 wild-type infectious cDNA clone. DAPW is a wild-type virus stock similar to the one used as a parent virus to generate 5 escape mutant viruses.

### FIG. 2

One-step growth curves of DAFL3 and mutant viruses on BHK-21 cells. The studies were performed as described under Materials and Methods. Results shown are the mean titers from two samples that were separately assayed from each time point.

### TABLE 3

<table>
<thead>
<tr>
<th>Virus</th>
<th>Titer (PFU/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAFL3</td>
<td>2.9 ( \times ) 10^4</td>
</tr>
<tr>
<td>DA-VP2-141 (Asn)</td>
<td>9.0 ( \times ) 10^3</td>
</tr>
<tr>
<td>DA-VP2-143 (Asp)</td>
<td>7.8 ( \times ) 10^3</td>
</tr>
<tr>
<td>DA-VP2-148 (Asp)</td>
<td>3.0 ( \times ) 10^4</td>
</tr>
<tr>
<td>DA-VP2-162 (Ser)</td>
<td>1.4 ( \times ) 10^4</td>
</tr>
<tr>
<td>DA-VP2-173 (Phe)</td>
<td>4.8 ( \times ) 10^3</td>
</tr>
</tbody>
</table>
TABLE 4
Pathological Findings Following Inoculation of Wild-Type and Mutant DA Viruses

<table>
<thead>
<tr>
<th>Virus</th>
<th>Mean ± SD</th>
<th>Number with white matter disease/total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAFL3</td>
<td>2.7 ± 0.6</td>
<td>14/15 (93)</td>
</tr>
<tr>
<td>DA-VP2-141 (Asn)</td>
<td>0.8 ± 0.6**</td>
<td>0/22 (0)</td>
</tr>
<tr>
<td>DA-VP2-143 (Asp)</td>
<td>2.0 ± 0.6*</td>
<td>7/9 (78)</td>
</tr>
<tr>
<td>DA-VP2-148 (Ala)</td>
<td>1.8 ± 0.9*</td>
<td>5/8 (63)</td>
</tr>
<tr>
<td>DA-VP2-162 (Ser)</td>
<td>2.3 ± 0.2</td>
<td>8/8 (100)</td>
</tr>
<tr>
<td>DA-VP2-173 (Phe)</td>
<td>0.3 ± 0.4**</td>
<td>0/8 (0)</td>
</tr>
</tbody>
</table>

* Pathology of animals graded according to Material and Methods. The number of animals with white matter disease represents those with a grade ≥2.
** P < 0.001 vs DAFL3.

Preparation of engineered wild-type and mutant viruses

Wild-type pDAFL3 or mutant pDAFL3 (see previous section) cDNA transcription templates were linearized by restriction endonuclease XbaI (Boehringer Mannheim Biochemicals, Indianapolis, IN), which digests 3′ to the terminus of the viral genomic cDNA construct. The cDNA was then transcribed with T7 RNA polymerase and the RNA product was transfected into BHK-21 cells, as previously published (Roos et al., 1989a). The resultant virus was plaque-purified twice and passed an additional two times in BHK-21 cells to produce a virus stock. The DAFL3 virus stock used in the present study was a wild-type virus generated from transfection of transcripts derived from pDAFL3. To confirm the presence of the mutation in the virus, RNA was extracted using RNAzol (Biotecx) and cDNA, prepared by RT-PCR, was analyzed by an GIBCO BRL dsDNA cycle sequencing kit.
Neutralization assay

Plaque-reduction neutralization tests were performed as previously described (Nitayaphan et al., 1985; Roos et al., 1989b). Approximately 100 plaque forming units (PFU) of parental or mutant virus were incubated for 1 hr at room temperature in an equal volume of various dilutions of ascites fluid or hybridoma supernatant containing the respective mAb or an irrelevant mAb that served as a negative control. The virus–antibody mixture was allowed to adsorb onto BHK-21 cells with rocking for 1 hr at 33°C. The cell monolayers were overlaid with 1% agarose and 5% fetal bovine serum in modified Eagle medium (GIBCO BRL) and incubated for 4–6 days at 33°C. After fixation and staining, plaques were counted. Neutralization was defined as a reduction in the number of plaques by greater than 50% of control. A neutralization escape mutant was defined as a virus that requires approximately 101.5-fold more mAb to be neutralized than the same amount of wild-type virus.

Animal inoculation

Weanling SJL/J mice (Jackson Laboratory, Bar Harbor, ME) were inoculated intracerebrally with 0.03 ml con-
taining $1 \times 10^7$ PFU/ml of virus except in the case of DAFL3 (1.5 $\times 10^7$ PFU/ml) and DA-VP2-141(Asn) (4.0 $\times 10^6$ PFU/ml) viruses. Mice were sacrificed at 1 and 6 weeks postinoculation (PI) and their brains and spinal cords were removed for virus assays or histopathology.

Quantitative pathologic analysis in spinal cords

Spinal cords from at least 8 mice from each group inoculated 6 weeks previously with either wild-type or mutant virus were fixed in 10% formalin and embedded in paraffin. Sections of spinal cord were stained with hematoxylin and eosin. The pathological findings were scored usually on at least five spinal cord sections from each mouse as follows: "-" no pathology; "+," meningitis with not more than one inflammatory infiltrate in the parenchyma examined and no disruption of the white matter; "+ +," moderate inflammation and white matter disruption (that is present in about half of the white matter parenchyma examined); "+ + +," severe inflammation and white matter disruption (that is present in about 75% of the white matter parenchyma examined). A mean score and standard deviation (SD) of the white matter disease was determined for each group of mice inoculated with wild-type or mutant virus by setting the grade of "-" through "+ + +" equivalent to 0 through 3." Statistical comparisons were performed according to the Mann–Whitney U test.

Analysis of virus growth

Viruses were tested for in vitro growth with one-step growth curves. Viruses were adsorbed onto petri dishes of BHK-21 cells for 1 hr at a multiplicity of infection of 10. The monolayers were washed and scraped at 3, 6, and 14 hr after infection. Cells and supernatants were assayed for infectivity by means of a plaque assay on BHK-21 cells.

Viruses were tested for in vivo growth by assaying virus infectivity in brains from three mice inoculated with wild-type or each of the mutant viruses 1 week PI. The brain was homogenized into a 20% solution in Hank's-buffered saline solution and assayed by means of plaque assay.

Analysis of viral genome persistence

To investigate virus persistence, RNA was extracted from the spinal cords of two or three mice from each group inoculated with either wild-type or mutant virus 6 weeks previously. An RT reaction was performed with a random hexamer primer (Promega, Madison, WI). The RT products were amplified by PCR with two primers that amplify the coding region of VP2 (5'-AATGCTTTTGCT-ACTATG, 3'-GGGGTTTACGGGCTGGAT). The conditions of the RT-PCR were as previously described (Zhang et al., 1995). To ensure that a similar amount of cDNA was generated in the RT reaction, PCR was performed on the same sample using hypoxanthine phosphoribosyl transferase (HPRT)-specific primers (Reiner et al., 1993).

RESULTS

DA VP2 141 is part of a neutralization site

To delineate the importance of VP2 141 as a neutralization site of DA virus, DAFL3 VP2 141 Lys was changed to Asn by site-directed mutagenesis, and the resultant virus, DA-VP2-141 (Asn), was tested in a plaque-reduction neutralization test. Several TMEV neutralizing mAbs that we have described (Nitayaphan et al., 1985) neutralized this mutant virus as efficiently as wild-type virus (data not shown); however, as shown in Table 1, DAmAb1 neutralized wild-type DAFL3 virus, but failed to neutralize DA-VP2-141 (Asn) mutant virus; similar results were obtained with another mutant virus separately derived from a different pDA-VP2-141 (Asn) cDNA clone. These results suggested that VP2 141 is part of an epitope of DAmAb1.

DAmAb1 escape mutant viruses have changes in the VP2 EF loop

To further define the location of a neutralization site of DAmAb1 in the VP 2 EF loop, we sequenced the whole coding region for VP2 from five DA isolates that had been grown in the presence of DAmAb1. We compared these sequences with the sequence from two wild-type viruses, DAPW and DAFL3 (Table 2). DAPW, a wild-type virus similar to the one used as the parent for the generation of the DAmAb1 escape mutant viruses, had evidence of nucleotides coding for both a Thr and Ala at VP2 148 and of both a Gly and Ser at VP2 162, suggesting a mixed population of virus; in contrast, DAFL3 virus had coding sequence for a Thr at VP2 148 and Gly at VP2 162. In comparison to DAFL3 virus, all five DAmAb1 escape mutant viruses had changes of VP2 148 from Thr to Ala and of VP2 162 from Gly to Ser; we suspect that these differences from DAFL3 virus were not important in resistance to DAmAb1 since they were also present in DAPW virus. When compared to DAPW and DAFL3 virus, four of five of the mutant viruses had a change in VP2 143 from Gly to Asp, while the fifth mutant had a VP2 173 Thr to Phe change (Table 2). The importance of all of these mutations with respect to virus neutralization was tested, as described below.

DAVP2 143 and DAVP2 173 are part of the DAmAb1 neutralization site

As noted above, there were differences in amino acids in the VP2 EF loop found in escape mutant vi-
Virus infectivity

The above findings were supported by studies with DAmAb1 escape mutant viruses. DAmAb1 escape mutant viruses did not have a specific defect in growth in vitro or in the brain.

The role of DA-VP2-141 and the surrounding region in disease phenotype

Wild-type and mutant DA viruses were inoculated intracerebrally into 3-week-old SJL/J mice, and animals were sacrificed at 6 weeks PI (Fig. 1, Table 4). As expected, all but 1 of 15 mice infected with wild-type DAF3 virus had moderate−severe pathological changes (greater than or equal to 2), with white matter disruption and lymphocyte infiltration of the spinal cord (Fig. 3a, Table 4). A similar white matter disease pathology was seen in most of the animals inoculated with DA-VP2-143 (Asp), DA-VP2-148 (Ala), and DA-VP2-162 (Ser) viruses (Fig. 1, Table 4); the mean degree of pathology was lower for the first two mutants than that seen with wild-type virus or DA-VP2-162 (Ser) virus (Table 4); however, it was clear that these mutant viruses were capable of producing significant white matter disease. These results were in marked contrast with those seen following inoculation with DA-VP2-141 (Asn) and DA-VP2-173 (Phe) viruses.

None of 22 mice inoculated with DA-VP2-141 (Asn) and none of 8 mice inoculated with DA-VP2-173 (Phe) virus had significant white matter pathology. Only occasional mice inoculated with these two mutant viruses had minimal meningitis or very infrequent small inflammatory cell infiltrates of the parenchyma (Table 4, Figs. 3b and 3c). The minimal white matter pathology seen following DA-VP2-141 (Asn) virus infection was of special note since animals were inoculated with more than 10-fold more PFU of this mutant virus than with the other viruses. Similar results were obtained from an additional two other DA-VP2-141 (Asn) mutant viruses, derived from separate cDNA clones.

Data from Epon-embedded sections of spinal cords was consistent with the above results. Mice inoculated with DAF3 and DA-VP2-143 (Asn) viruses demonstrated demyelination. Small areas of infiltrated macrophages and inflammatory cells from animals inoculated with DA-VP2-141 had evidence of some demyelination; however, these areas were extremely infrequent. Two of two DA-VP2-173 (Phe)-inoculated mice failed to demonstrate any evidence of demyelination, presumably because these regions were so difficult to find.

The above findings were supported by studies with DAmAb1 escape mutant viruses. DAmAb1 escape mutant virus 5, which has VP2 173 (Phe) (as well as VP2 148 [Ala] and 162 [Ser]) failed to demonstrate any white matter disease at 6 weeks PI, while DAmAb1 escape mutant viruses 1–4, which have VP2 143 (Asp) (as well as VP2 148 [Ala], and 162 [Ser]) demonstrated white matter destruction and meningitis (data not shown).

In order to determine whether virus persisted we analyzed spinal cords from inoculated animals for the presence of viral genome by RT-PCR. Viral genome persisted in the spinal cord of mice inoculated by DA-VP2-141 (Asn), DA-VP2-143 (Asp), DA-VP2-148 (Ala), DA-VP2-162 (Ser), (Fig. 4); and DA wild-type viruses, the presence of the appropriate mutation in the viral genome-amplified products was confirmed by sequencing studies. In contrast, RNA extracted from the spinal cords of mice inoculated 6 weeks previously with DA-VP2-173 (Phe) virus showed no evidence of viral genome. The absence of
amplification was not related to the primers used for PCR since the same primers efficiently amplified tissue culture stocks of DA-VP2-173 (Phe) virus as well as brain tissue from mice infected 1 week previously with the mutant.

**DISCUSSION**

TMEV is of special interest to virologists because of its unusual disease phenotype. TO subgroup strains, such as DA, persist in the CNS for the whole life of the mouse and appear to have a restricted infection in glial cells and macrophages. In addition, DA-induced demyelinating disease serves as an experimental model of MS: both disease processes have a similar white matter pathology, and the immune system appears to contribute to demyelination in both. A goal of our work is to identify TMEV disease determinants and to characterize the anti-TMEV immune response in order to better understand the pathogenesis of TMEV-induced disease, as well as MS.

Previous investigations of DA virus mutants generated in the presence of various neutralizing mAbs have proven valuable in the identification of neutralizing sites on the virus as well as molecular determinants of TMEV-induced disease. We now show that DA amino acid 141 Lys, 143 Gly, and 173 Thr in the VP2 EF loop are part of a neutralization site. We also tested isolates of GDVII virus that were grown in the presence of GDVII mAb4 and mAb6 (both of which share the same neutralizing site on GDVII subgroup strains (Nitayaphan et al., 1985)) for the presence of a mutation in the EF loop (data not shown). All three GDVII escape mutants that were tested had a change in wild-type GDVII VP2 142 from Ser to Phe, suggesting that, like DAmAb1, GDVII mAb4/6 have a neutralization site on the TMEV VP2 EF loop; however, different amino acid residues presumably confer TO or GDVII subgroup specificity on these mAbs. In the case of poliovirus type 1, VP2 142 in the EF loop is similarly part of a neutralization site (and affects mouse neurovirulence) (Couderc et al., 1991).

Although three DA residues are part of the DAmAb1 neutralizing site in the EF loop, they do not have a similar role in determining disease phenotype. Mice inoculated with DA-VP2-143 (Asp) virus showed white matter pathology with virus persistence similar to that seen in mice inoculated with wild-type virus. DA-VP2-173 (Phe) virus neither persisted nor induced demyelination at 6 weeks PI. DA-VP2-141 (Asn) virus persisted in the spinal cord for 6 weeks PI; however, this mutant virus induced significantly less late white matter pathology than wild-type virus. The persistence of DA-VP2-141 (Asn) in the absence of significant white matter disease is surprising; it may be that there is an insufficient amount of the virus to induce demyelination or that the virus is located in different neural cells than is the case with wild-type virus. The diversity and complexity of disease phenotype presumably reflects the varied effects of the mutated amino acid residue on the three-dimensional structure of the viral capsid. This variation of disease phenotype may be responsible for our finding that DA-VP2-143 (Asp) and DA-VP2-148 (Ala) viruses produce white matter pathology that is significant in degree, but attenuated compared to that seen with wild-type virus. Interestingly, the particular amino acid residues that constitute poliovirus type 1 neutralization antigenic site 1 (VP1 94-102 in the BC loop) also have a very variable effect on mouse neurovirulence (Couderc et al., 1991).

In contrast to DA-VP2-141 (Asn) virus, TMDA virus (which is derived from an infectious cDNA clone of DA141 by Brahic and colleagues (Jarousse et al., 1994)) is reported to have demyelinating activity although it also has the critical Asn at VP2. Presumably, DA-VP2-141 (Asn) and TMDA have another sequence difference(s) that modulates the demyelinating activity. This difference may reside in the 1B-2A segment, as suggested by the fact that the demyelinating activity of GD1B-2A/DAFL3 recombinant virus appears to be modulated by an interaction of this segment with the VP2 141 amino acid residue.

Our results are more readily interpreted in the context of the known three-dimensional structure of the DA virus.

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**FIG. 5.** Mutations found in DA escape mutant viruses resistant to neutralizing mAbs mapped onto the X-ray crystal structure of the capsid of DA strain. (A) Color-coded spheres mark the locations of the X-ray crystal structure of the TMEV capsid. The surface features of the virus are highlighted by coloring the trace in a continuous spectrum from dark blue (low radius) to white (high radius). Features at a radius below 135 Å disappear into the black background. The spheres represent amino acid residues that have been mutated to escape neutralization by TMEV mAbs. The colors of the labels define the color-coding of the spheres. The larger spheres mark sites where single amino acid substitutions have been shown to attenuate the ability of DA virus to persist and cause demyelination, while mutation at the sites marked by the smaller spheres do not (see text). Note that residue 268 of VP1 is not marked with a large sphere in this panel or in B because its precise location is unknown [since the C-terminus of VP1 (residues 257–274) is disordered and therefore not visible in X-ray structure]; for this reason, the last visible residue of VP1 (256) is marked instead. (B) Close-up stereo view shows the sites mutated in DA escape mutant viruses resistant to TMEV neutralizing mAbs in one of the 60 protomers comprising the capsid. The color coding of the spheres is as in A, with the larger spheres marking sites where individual amino acid substitutions can affect the ability of the virus to persist and demyelinate. Each site is highly exposed on the capsid surface. VP 101 is located in the second part of the CD loop of VP1. All of the VP2 residues are in the EF loop of VP2. VP 2141 is located at each point of the prominent star-shaped plateaus at the fivefold symmetry axes (A). Residues 143 and 148 of VP1 are also near the tips of the star. Residue 173 of VP2 and residue 101 of VP1 are located near each other at the edge of the plateau, overlooking the large depression (pit) spanning the twofold axis. Residue 162 of VP2 is located at a lower radius, about halfway down the slope connecting the bottom of the depression and the edge of the plateau.
Neutralizing antibody determinants of picornaviruses are generally, but not always, located on the outer surface of the capsid (Page et al., 1985; Li et al., 1994). Not surprisingly, all of the amino acids presently implicated in DA virus neutralization (reviewed in Roos and Casteel, 1992) are also exposed on the capsid surface where they are readily accessible to antibodies: VP1 101 is in the CD loop of VP1; VP2 141, 143, and 173 are in the EF loop of VP2; and VP1 268 is in the disordered carboxyl terminus of VP1, which, although not visible in the crystallographic electron density map, is definitely exposed on the surface (Fig. 5).

All of the residues of DA that have been identified as neutralization epitopes cluster around a depression between the arms of the star-shaped plateaus that dominate the surface of the capsid (Fig. 5A). This region, which consists mainly of the exposed surface of VP3, has been called the “pit” by Luo et al. (1992). The pit is actually part of a larger depression that spans the twofold icosahedral symmetry axes (Grant et al., 1992). The pit region has been proposed to be the TMEV receptor binding site based on the previously observed clustering of important sites in the area (Grant et al., 1992; Jarousse et al., 1994; Luo et al., 1992). Although there is no direct evidence for the interaction of the cellular receptor with the bottom of the pit, the location of VP1 101 and VP2 141 and 173 at the edge of the plateau overlooking the pit and the emergence of the disordered carboxyl terminus of VP1 (including VP1 268) from the bottom of the pit coupled with data demonstrating that mutations in these residues induce a change in disease phenotype (Zurbriggen et al., 1991; Roos et al., 1989b) suggest that the structure in this area is critically important in determining the pathogenesis of TMEV infection. Structural alterations in this area allow the virus to escape neutralization and presumably also disrupt virus–receptor interactions that control tissue tropism in the infected CNS. Similarly, in poliovirus, amino acid residues that are known to constitute neutralization sites also play a role in the recognition and binding of the receptor (Colston et al., 1995; Couderc et al., 1994, 1991; Martin et al., 1991; Murray et al., 1988). The imperfect congruence between TMEV sites critical in neutralization and in disease phenotype suggests that the antibody-binding sites only partly overlap those that affect receptor binding.

ACKNOWLEDGMENTS

The secretarial assistance of Ms. Ellie Orr is appreciated. This work was supported by grants from the National Multiple Sclerosis Society and the National Institutes of Health.

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