Chimeric cDNA Studies of Theiler’s Murine Encephalomyelitis Virus Neurovirulence

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Strain GDVII and other members of the GDVII subgroup of Theiler’s murine encephalomyelitis virus are highly neurovirulent and rapidly fatal, while strain DA and other members of the TO subgroup produce a chronic, demyelinating disease. GDVII/DA chimeric cDNA studies suggest that a major neurovirulence determinant is within the GDVII 1B through 1D capsid protein coding region, although the additional presence of upstream GDVII sequences, including the 3’ untranslated region, contribute to full neurovirulence. Our studies indicate that there are limitations in precisely delineating neurovirulence determinants with chimeric cDNAs between evolutionarily diverged viruses, such as GDVII and DA.

The term Theiler’s murine encephalomyelitis virus (TMEV) refers to picornavirus strains most closely related to the cardiovirus genus that can be separated into two subgroups on the basis of their biological activities (7, 8; for a review, see reference 13). After inoculation into weanling mice, strain GDVII and other members of the GDVII subgroup of TMEV are extremely neurovirulent and rapidly fatal. In contrast, strain DA and other members of the TO subgroup produce a chronic persistent demyelinating infection that resembles multiple sclerosis.

One of our main goals is to define the molecular determinants of TMEV-induced disease and the mechanisms involved. Despite the markedly different biological activities of strains from the two TMEV subgroups, different strains have approximately 90% identity at the nucleotide level and 95% identity at the amino acid level. With this in mind, infectious clones of DA (pDAFL3) and GDVII (pGDVIIFL2) have been prepared and chimeric cDNAs and recombinant viruses have been generated (1–3, 9, 12, 14, 16, 18). The present study attempts a finer delineation of TMEV neurovirulence determinants.

Chimeric cDNA clones, constructed by replacing part of pDAFL3 with segments from pGDVIIFL2, are named by first listing 5’ and 3’ junctions of the GDVII segment, then adding a slash, and finally adding DAFL3. Virus derived by transfection of GDVIIFL2 was fully neurovirulent, with a 50% lethal dose (LDso) of 0.7 PFU, while transfection-derived DAFL3 virus was not neurovirulent (LDso > 106 PFU) (Fig. 1). As previously published (2), all the recombinant viruses shown in Fig. 1 failed to kill mice unless they contained the 1B to 2C segment (1B-2C) from GDVII. Although GDVII 1B-2C was essential for neurovirulence, virus that contained only this GDVII segment did not achieve the full neurovirulence of the GDVII parental strain (GD1B-2C/DAFL3 [LDso = 102-7 PFU] versus GDFL2 [LDso = 0.7 PFU]). The addition of GDVII sequence 5’ to 1B-2C (GD5’-2C/DAFL3 virus) conferred full neurovirulence on the virus (LDso = 1 PFU), demonstrating that neurovirulence is multigenic and that certain regions have more effect than others. Despite the full neurovirulence of GD5’-2C/DAFL3 virus, the addition of GDVII sequence 3’ to 1B-2C (GD1B-3/DAFL3 virus) also enhanced the neurovirulence of GD1B-2C/DAFL3 virus, suggesting a redundancy of neurovirulence determinants, i.e., several regions of the genome can lead to equivalent degrees of neurovirulence. Redundancy is not unexpected given the likelihood that GDVII virus, under evolutionary pressure, might develop a variety of means to kill a mouse. This redundancy serves to complicate the identification of neurovirulence determinants by means of a chimeric GDVII/DA approach.

To further delineate key neurovirulence sequences, we divided GDVII 1B-2C into two pieces and separately substituted each segment into pDAFL3, constructing pGD1B-2A/DAFL3 and pGD2A-2C/DAFL3. Surprisingly, neither recombinant virus was significantly neurovirulent (Fig. 2A). These findings were observed with multiple clones, suggesting that the loss of virulence did not result from an unintended attenuating mutation introduced during the genetic construction. As additional confirmation of this point, the DA 2A-2C segment in GD1B-2A/DAFL3 chimeric cDNA (that had generated attenuated GD1B-2A/DAFL3 virus) was substituted with the equivalent GDVII segment; the resultant GD1B-2C/DAFL3 virus was as neurovirulent as prototypic GD1B-2C/DAFL3 virus.

We considered two possible explanations for these findings. (i) The presence of chimeric 2A attenuates GD1B-2A/DAFL3 and GD2A-2C/DAFL3 viruses. (ii) Interactions between GDVII genes or gene products that are critical for neurovirulence and intact in GD1B-2C/DAFL3 virus are disrupted in the attenuated constructs. To investigate these issues, we constructed pGDL-2A/DAFL3 with the same AarII junction in the 2A coding region as in the attenuated viruses. GDL-2A/DAFL3 virus was as neurovirulent as GD1B-2C/DAFL3 (Fig. 2B), indicating that chimeric 2A is not the explanation for attenuation of GD1B-2A/DAFL3 and GD2A-2C/DAFL3 viruses. The decreased growth kinetics of GD1B-2A/DAFL3 and GD2A-2C/DAFL3 viruses (see below) supported the hypothesis that interactions of GDVII genes or gene products critical for neurovirulence were disrupted in these viruses.

We presumed that neurovirulence determinants in GDVII L-2A (and GDVII 1B-2A) were present upstream from P2 and not in 2A since the part of 2A contributed by GDVII contained only two amino acids different from DA. To more carefully investigate this, we engineered a silent Xhol site at the 3’ end of the 1D coding region of GDVII and DA to generate chimeras with a “perfect” GDVII/DA 1D/2A junc-

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This enabled us to exactly transfer a segment from the GDVII 5' terminus to the XhoI site to produce GDS'-XhoI/DAFL3. The latter virus is as neurovirulent as parental GDVII virus (Fig. 3A), indicating that GDVII sequences upstream from P2 are sufficient for full neurovirulence.

The GDVII 5' untranslated region (5' UTR) had a clear effect on neurovirulence when certain GDVII downstream sequences were also present, since GDS'-XhoI/DAFL3 virus (LD50 = 0.7 PFU, like GDFL2 virus) is 20 to 100 times more neurovirulent than the most neurovirulent recombinant that does not contain the 5' UTR, GDL-2A/DAFL3 (LD50 = 10^{1.2} PFU). In addition, even a single nucleotide change in the TMEV 5' UTR can dramatically attenuate the virus (11), as is true for other picornaviruses (10). It should be noted, however, that most of the difference in neurovirulence among GDVII and TO subgroup strains depends on the presence of GDVII sequence downstream from the 5' UTR rather than the presence of the GDVII 5' UTR, i.e., the differences in the LD50s of neurovirulent recombinant viruses that contain the GDVII 5' UTR (LD50 = 0.7 PFU) and lack it (LD50 = 10^{1.2} PFU) are minimal compared with the difference between the LD50s of GDVII (LD50 = 0.7 PFU) and DA (LD50 > 10^6 PFU) strains.

To more finely delineate sequences downstream from the 5' UTR critical for neurovirulence, we prepared additional constructs with the downstream engineered XhoI P1-P2 junction site and with decreasing upstream margins of the GDVII segments (Fig. 3B). GDL-XhoI/DAFL3 virus, which contains GDVII sequences from L to the engineered XhoI, had an LD50 of 10^{3.4} PFU. The LD50 of GDIA-XhoI/DAFL3, which contains a perfect junction from GDVII 1A(VP4) through XhoI and contains only GDVII P1 coding sequence, was approximately 10^{2.8} PFU, suggesting that the GDVII capsid proteins are not sufficient to produce full neurovirulence when substituted into DA. The viruses generated from GDB1-XhoI/DAFL3 and GDB1-XhoI/DAFL3 were about as attenuated as GDB1-2A/DAFL3 virus. Despite this latter result, the LD50s of GDB1-2C/DAFL3, GDL-XhoI/DAFL3, and GDIA-XhoI/DAFL3 suggest that significant neurovirulence determinants (presumably disrupted in GDB1-XhoI/DAFL3 and GDB1-XhoI/DAFL3 viruses) are included in GDVII regions 1B through 1D.

We suspect that there are varied mechanisms by which a disruption of interactions between GDVII genes or gene products that are critical for neurovirulence can lead to the apparent attenuation of some recombinant viruses that may actually contain neurovirulence determinants. Attenuation of some recombinant viruses which contain parts of GDVII P1 (e.g., GDB1-XhoI/DAFL3 may result from interference with virion assembly or receptor binding because of chimeric...
(disrupted) capsid regions. In some cases, chimeric L-P1-2A and chimeric P2 may lead to impaired protein processing and attenuation of other recombinants. Occasional chimeric cDNA constructs, such as GD1C-XhoI/DAFL3, were not infectious at all in vitro despite repeated attempts to prepare them, probably because these constructs had a more drastic disturbance in genome interactions that are critical for infectivity.

We investigated the in vitro growth properties of selected recombinant viruses. GDVIIIFL2 virus grew to higher levels than DAFL3 virus at 7 and 9 h postinfection (Table 1), and, as previously reported (12), GD1B-2C/DAFL3 grew to levels between those seen with GDVIIIFL2 and DAFL3 viruses. GD1B-2A/DAFL3 and GD2A-2C/DAFL3 viruses, however, grew to levels even lower than DAFL3 virus at both time points, perhaps reflecting a disruption of interactions critical for efficient in vitro growth (as well as neurovirulence). In these cases, neurovirulence seemed to correlate with efficient in vitro growth. On the other hand, recombinant viruses GD1B-XhoI/DAFL3 and GD1D-XhoI/DAFL3 grew to reasonably high levels, approaching those seen with GDVIIIFL2 virus (at 7 h postinfection) despite their attenuated phenotype. The neurovirulence phenotype has similarly been found to correlate imperfectly with tissue culture growth in the case of other picornaviruses and nonpicorna-

viruses (5, 10); neurovirulence may correlate better with nervous system virus growth, as previously suggested (12).

Difficulties related to chimeric cDNA studies appear to be less of a problem with respect to poliovirus intratypic recombinant viruses than for GDVII and TO, probably because Sabin strains were derived after relatively few passages of the parental strain (with the subsequent generation of a limited number of point mutations), while there is substantial evolutionary divergence of the GDVII and TO viruses.

FIG. 2. Chimeric cDNAs generated to delineate sequences important in the neurovirulence of the GDVII 1B-2C segment. (A) The GDVII 1B-2C segment (in GD 1B-2C/DAFL3 virus) contains a major neurovirulence determinant; however, there is surprisingly little or no neurovirulence when either GDVII 1B-2A or GDVII 2A-2C is separately substituted into the DA genome. (B) GDL-2A/DAFL3 virus is similar in neurovirulence to GD1B-2C/DAFL3, indicating that the attenuation of GD1B-2A/DAFL3 virus is not due to a chimeric 2A.

**TABLE 1. Growth of virus at 7 and 9 h after infection of BHK-21 cells**

<table>
<thead>
<tr>
<th>Virus</th>
<th>Virus titer (% of DAFL3)* at:</th>
<th>7 h postinfection</th>
<th>9 h postinfection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>DAFL3</td>
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<td>$10^{8.3}$ (100)</td>
<td>$10^{7.9}$ (100)</td>
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<td>GDVIIIFL2</td>
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<td>$10^{8.6}$ (195)</td>
<td>$10^{8.0}$ (141)</td>
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<tr>
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<td>$10^{7.5}$ (44)</td>
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<tr>
<td>GD1B-2A/DAFL3</td>
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<td>$10^{7.3}$ (9)</td>
<td>$10^{7.3}$ (35)</td>
</tr>
</tbody>
</table>

* Expressed as PFU per milliliter and percentage of the titer of DAFL3 at the specific time point studied.
subgroups. It may be that other approaches besides GD-VII/DA chimeric cDNA studies will be valuable in the identification of disease determinants, such as the use of intrastrain chimeras between a wild-type parent and a pas saged strain with an altered disease phenotype. We are presently studying chimeras between tissue culture-passed strain DA (which is normally attenuated) and suckling mouse brain-passed DA (which has heightened neurovirulence [6, 8, 15, 17]).

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