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The effect of Theiler's murine encephalomyelitis virus (TMEV) VP1 carboxyl region on the virus-induced central nervous system disease

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Members of the Theiler's murine encephalomyelitis virus GDVII subgroup, which includes GDVII strain, are highly neurovirulent and induce a rapidly fatal polioencephalomyelitis. By contrast, Theiler's original subgroup members, which includes DA strain, are not as neurovirulent, and produce a chronic, demyelinating disease with virus persistence. We investigated the importance of the carboxyl region of the capsid protein VP1 in TMEV-induced disease since a trypsin-cleavable immunodominant neutralization epitope is situated in the VP1 carboxyl region, and since this region is believed to lie adjacent to the putative receptor binding site. The present studies support the role of DA VP1 residue 268 (and the aligned GDVII VP1 270) in Theiler's murine encephalomyelitis virus-induced CNS disease; however, the effect of this residue varies depending on its context: mutation of DA VP1 268 attenuates demyelination; mutation of GDVII VP1 270 in a GDVII/DA recombinant virus has no effect on demyelination but reduces early deaths (neurovirulence); mutation of GDVII VP1 270 in GDVII virus has no effect on neurovirulence. These data suggest that DA VP1 268/GDVII VP1 270 are not functionally equivalent and that a residue in recombinant viruses can differ in function from the same residue situated in a parental strain. Additional mutagenesis studies suggest that: the trypsin cleavage site of TMEV, which affects virus viability, is located at the lysine at DA VP1 261 (GDVII VP1 263); GDVII VP1 276, the predicted carboxyl terminus of VP1, affects VP1/2A processing and virus infectivity.

Keywords: Theiler's virus; picornavirus; demyelination; neurovirulence; molecular pathogenesis; multiple sclerosis

Introduction

Theiler's murine encephalomyelitis virus (TMEV), a member of the picornaviridae family, is a common, generally asymptomatic enteric mouse virus which infrequently causes central nervous system (CNS) disease (reviewed by Roos and Casteel, 1992). CNS disease is consistently produced, however, following intracerebral inoculation of weanling mice with TMEV. Based on different disease phenotypes, and antigenic properties, TMEV is divided into two subgroups. Members of Theiler's original (TO) subgroup, including DA strain, cause a biphasic disease with early motor neuron disease followed by chronic demyelination. This chronic demyelinating disease is an excellent experimental model for multiple sclerosis. In contrast, members of the GDVII subgroup, including GDVII strain, neither persist nor demyelinate, but instead cause an acute, fatal gray-matter neuronal infection.

The mechanisms responsible for the differences in biological properties between the two subgroups are unknown. We are interested in determining regions of the virus which may affect disease phenotype by changing interactions with cell receptors or influencing the antigenic response to the virus. The carboxyl region of TMEV VP1 is of special interest because trypsin-sensitive neutralization epitopes are located in this region (Nitayaphan *et al*, 1985a, b) and because X-ray crystallographic studies of DA suggest that the carboxyl end of DA VP1 forms a mobile region which can pass over the rim of the putative receptor binding site, the 'pit' (Luo *et al*, 1987; Grant *et al*, 1992); these latter

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observations suggest that the carboxyl region of VP1 may influence the immune response (and thereby affect virus clearance) and/or influence the binding to cell receptors (and thereby affect tropism). In the present study we mutagenize VP1 amino acid residues and identify determinants critical for neutralization, trypsin sensitivity, and processing at the VP1/2A junction. We also demonstrate that the effect of a particular residue in a parental strain may be different from its effect in a recombinant virus.

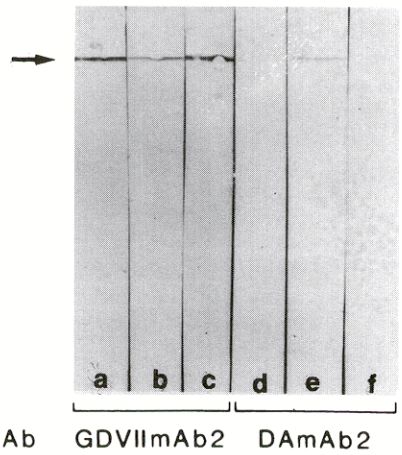

Figure 1 Western blot of proteins of DA-VP1-268 virus (lanes a and d), wild-type DA virus (lanes b and e), and the DA mAb2 escape-mutant virus (lanes c and f) immunostained with GDVII mAb2 (lanes a–c) and DA mAb2 (lanes d–f). See details of the methods in 'Materials and methods.' DA mAb2 immunostains VP1 (arrow) of wild-type DA but not the DA mAb2-escape-mutant virus or the genetically engineered mutated virus, DA-VP1-268. Note that GDVII mAb2 is a VP1-specific mAb with an epitope distinct from that of DA mAb2 and, therefore, immunostains VP1 of wild-type and the mutated DA.

Results

The presence of VP1 268 Phe in DA virus attenuates demyelination

We showed previously that a virus resistant to neutralization by a VP1- and TO-subgroup-specific neutralizing mAb, DA mAb2, had a mutation in the VP1 carboxyl region, changing DA residue VP1 268 from Val to Phe (Ohara *et al*, 1988). When inoculated into weanling mice, this virus caused little or no demyelination compared to wild-type DA virus (Roos *et al*, 1989). In order to ensure that this single VP1 mutation was responsible for resistance to DA mAb2 neutralization and for the change in disease phenotype, we prepared and characterized virus that was genetically engineered to carry the same mutation.

In order to produce virus with a change in residue 268 from Val to Phe, we mutated the full-length DA cDNA clone, pDAFL3, at nucleotide (N) 3805 from G to T. The resultant virus, DA-VP1-268, was tested in a Western blot against DA mAb2. As expected, DA mAb2 immunostained VP1 of wild-type DA virus (Figure 1, lane e), but failed to immunostain DA-VP1-268 virus (Figure 1, lane d) just as it failed to immunostain the DA mAb2 escape mutant virus (Figure 1, lane f). GDVII mAb2 is a VP1-specific mAb with an epitope distinct from that of DA mAb2 and, therefore, immunostains VP1 of wild-type (Figure 1, lane b) and the two DA mutants (Figure 1, lanes a,c).

In order to test the effect of DA-VP1-268 virus on the demyelinating disease, weanling mice were inoculated, and spinal cord sections were examined at 6 weeks or 3 months post-inoculation (Table 1). Of the 30 mice observed for 6 or more weeks following inoculation with DA-VP1-268 virus, only two showed signs of any spinal cord pathology (Figure 2a), and this pathology was minimal, eg, one small focus of meningitis. By contrast, 37 of 39 mice observed for 6 or more weeks following inoculation

Table 1 Pathological findings after infection with DA wild-type virus or DA-VP1-268 virus[a]

Virus	*Titer (PFU ml^{-1})*	*Postinoculation interval (no. affected/no. inoculated)*					
		12 days	*14days*	*21 days*	*4 wks*	*6 wks*	*3 months*
DA wild type	3.2×10^7	ND[b]	ND	3/3	ND	ND	13/13
	8.0×10^6	5/5	7/7	ND	ND	ND	8/10
	4.0×10^6	5/5	7/7	ND	ND	3/3	13/13
DA-VP1-268[c]	2.4×10^7	4/5	6/7	ND	0/2	1/5	ND
	1.8×10^6	3/5	ND	0/3	ND	ND	1/25

[a]The pathological findings that were evaluated consisted of meningitis and gray-matter inflammation in animals inoculated ≤ 21 days post-infection and white matter inflammation and demyelination ≥ 4 weeks post-infection
[b] ND, Not done
[c] DA-VP1-268 virus was engineered through site-specific mutagenesis to have the same change in VP1 residue 268 as DAmAb2 escape-mutant viruses

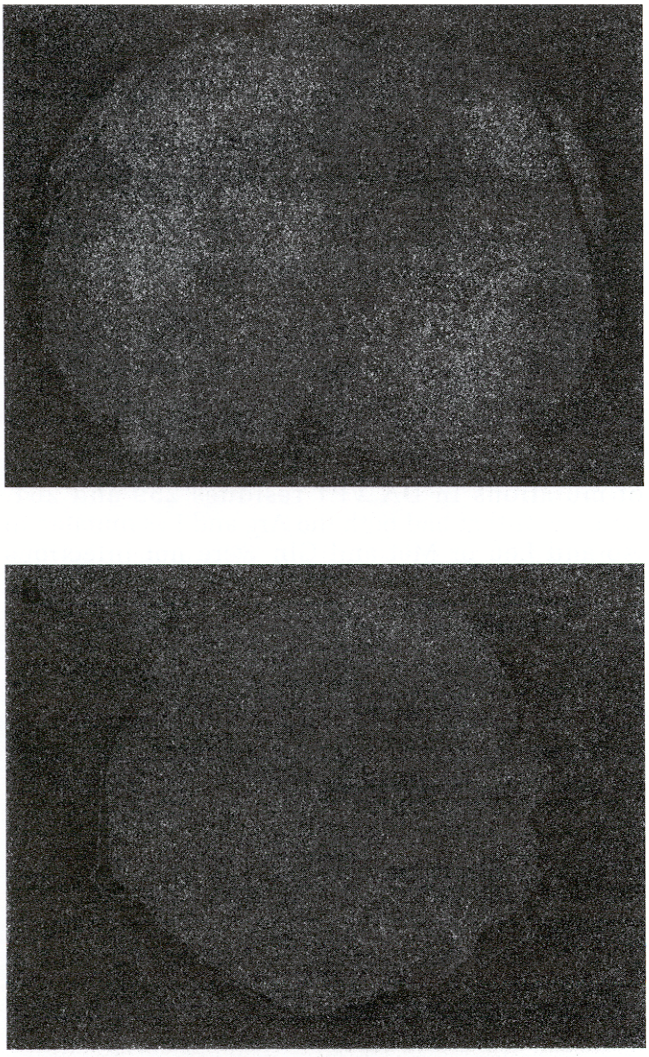

Figure 2 Histopathology of mouse spinal cords harvested 3 months after intracerebral inoculation with wild type DA or DA-VP1-268 virus. (a) The spinal cord from a representative mouse inoculated with DA-VP1-268 virus shows no histopathology. (Hematoxylin-eosin stained paraffin section; magnification, ×20). (b) The spinal cord from a representative mouse inoculated with wild-type DA virus which demonstrates an accumulation of inflammatory cells and extensive myelin loss. (Hematoxylin-eosin stained paraffin section; magnification, × 16).

with DA virus at three different titers showed prominent pathology, which included inflammatory cells and disrupted myelin (Figure 2b). These results are similar to those obtained after inoculation with the DA mAb2 neutralization-resistant escape mutant (Roos *et al*, 1989) and support the critical role of DA VP1 268 in the late inflammatory demyelinating disease.

The presence of VP1 270 phenylalanine in GD1B-2C/DAFL3 virus affects neurovirulence but not demyelination

When sequences at the VP1 carboxyl end of DA and GDVII are compared, the most carboxyl-terminal 14 residues are identical, including DA residue 268. Residue 268 is aligned with GDVII VP1 residue 270, but numbered differently, because of a six nucleotide insertion in the upstream DA VP1 coding region. Although GDVII is generally considered a non-demyelinating virus, recombinant virus studies involving replacement of parts of the GDVII genome with DA sequence suggest that GDVII has determinants for demyelination that are obscured by the rapid fatality induced by GDVII (Fu *et al*, 1990a). Therefore, in order to test the effect of GDVII VP1 270 on demyelinating activity, we mutated GD1B-2C/DAFL3 recombinant virus which is known to induce demyelination; this recombinant virus contains most of the coding region for GDVII capsid proteins including VP1 as well as several non-structural proteins substituted in the DA genome.

For the reasons noted above, we mutated pGD1B-2C/DAFL3 VP1 residue 270 from Val to Phe to produce GD1B-2C (VP1 270)/DAFL3 virus. Three months after inoculation with GD1B-2C (VP1 270)/DAFL3, spinal cord sections from all 13 surviving animals showed a degree of inflammation and demyelination similar to that seen in the one surviving mouse inoculated with wild-type GD1B-2C/DAFL3 virus-inoculated mice as well as in mice inoculated with GD1B-2C/DAFL3 virus as described

Table 2 Mortality and pathology following infection with GD1B-2C/DAFL3 and GD1B-2C (VP1 270)/DAFL3 viruses

Virus	*Titer (PFU ml^{-1})*	*Postinoculation interval (no. affected/no. inoculated)*	
		1 month[a]	*3 months*[b]
GD1B-2C/DAFL3	2.2×10^6	5/5	NA[c]
	2.2×10^5	4/5	1/1
GD1B-2C (VP1 270)/ DAFL3	1.3×10^6	1/5	4/4
	1.3×10^5	0/5	5/5
	1.3×10^4	1/5	4/4

[a] No. deaths/no. inoculated
[b] Histopathology includes inflammatory infiltrates and demyelination
[c] NA, Not applicable since there were no survivors after 1 month that could be tested

in previously published studies (Fu *et al*, 1990a; Rodriguez and Roos, 1992), ie, the presence of GDVII VP1 270 valine to phenylalanine in GD1B-2C/DAFL3 did not decrease demyelination (Table 2).

We also determined the effect of this mutation on the neurovirulence of the virus (Table 2). The LD_{50} for GD1B-2C (VP1 270)/DAFL3 was > 3.9×10^4 PFU. In contrast, the LD_{50} for mice inoculated with GD1B-2C/DAFL3 virus in this experiment was less than the least amount of virus inoculated, 6.6×10^3 PFU; in previous experiments involving smaller amounts of virus, the LD_{50} for GD1B-2C/DAFL3 virus was 2×10^2 PFU. Thus, a Phe rather than Val in VP1 270 of GD1B-2C/DAFL3 virus attenuates neurovirulence but does not affect demyelination.

The presence of VP1 270 phenylalanine in GDVII virus does not affect neurovirulence

Since GD1B-2C (VP1 270)/DA virus had an attenuation of neurovirulence compared to the unmutated recombinant virus (Fu *et al*, 1990b), we questioned whether the presence of this same mutation in GDVII virus would also have an attenuating effect on neurovirulence. Analysis of the results of intracerebral inoculation (Table 3) showed that the LD_{50} for GD-VP1-270 was < 12 PFU. Since GDVII wild-type virus has an LD_{50} of 0.7 PFU (Fu *et al*, 1990b), the presence of Phe VP1 270 in GDVII virus has no significant effect on neurovirulence; this result contrasts with the attenuation seen following inoculation with GD1B-2C (VP1 270)/DAFL3.

At 3 months following inoculation with GD-VP1-270, we examined mice for demyelination (Table 3). The nine survivors (that had received small amounts of virus) had no evidence of demyelination, as is true for the few survivors following GDVII virus infection (Fu *et al*, 1990a). These results suggest that demyelination may depend on infection with relatively large amounts of virus which, in the case of GD-VP1-270 and GDVII virus, is lethal.

DA VP1 residue 261 (GDVII 263) lysine is the probable site of trypsin cleavage and is necessary for virus viability

Trypsin treatment of DA or GDVII virus results in loss of about 2000 daltons from the carboxyl end of VP1 (Nitayaphan *et al*, 1985b) and disruption of the epitopes for the VP1-specific neutralizing monoclonal antibodies, DA mAb2 and GDVII mAb1/2 (Nitayaphan *et al*, 1985b). There are two possible sites for trypsin cleavage in the carboxyl region of DA VP1, an arginine at residue 259 and a lysine at residue 261. We were interested in identifying which of these two residues is the site for trypsin cleavage of DA and in determining the consequences of trypsin cleavage, especially in relation to DA-induced demyelination. We produced a number of mutations in DA VP1 residues 259 and 261. Constructs that had both the Arg and Lys mutated to Iso and Leu or Met and Gln were not infectious (Figure 3). The lack of infectivity was presumably related to the critical importance of one or both of these residues, and not due to an error in the construction of the clones since: multiple constructs were not infectious; the mutated nucleotides and the surrounding area were sequenced and found to be correct; translation of RNA transcripts derived from these clones demonstrated the correct reading frame (data not shown), and a construct in which

Table 3 Mortality and pathology following infection with GD-VP1-270 virus

Virus	*Titer (PFU ml^{-1})*	*Postinoculation interval (no. affected/no. inoculated)*	
		1 month[a]	*3 months*[b]
GD-VP1-270	4.0×10^3	5/5	NA[c]
	4.0×10^2	5/5	NA
	4.0×10^1	1/5	0/4
	4.0×10^0	0/5	0/5

[a] No. deaths/no. inoculated
[b] Histopathology includes inflammatory infiltrates and demyelination
[c] NA, Not applicable since there were no survivors after 1 month that could be tested

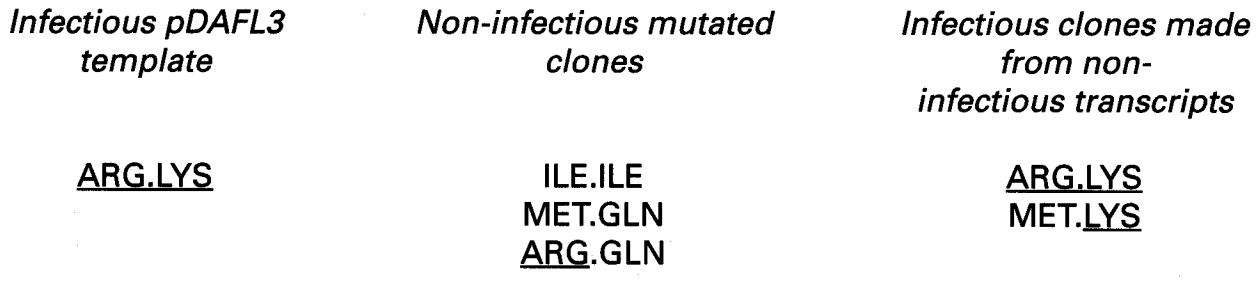

Figure 3 Infectivity of constructs with varied amino acids at two putative trypsin cleavage sites, DA VP1 residues 259 and 261. Wild-type (WT) residues are underlined. Note that constructs in which DA VP1 residue 261 is mutated are non-infectious.

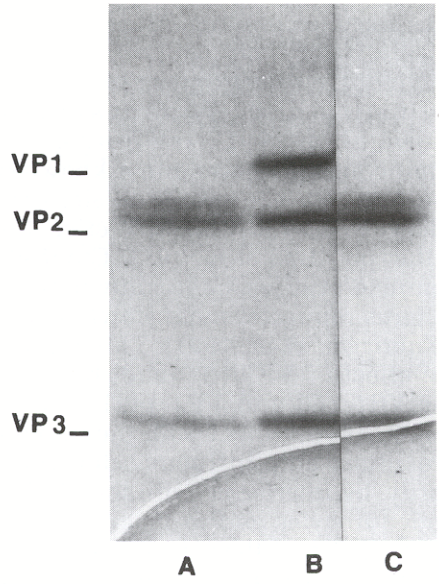

Figure 4 12.5% SDS-polyacrylamide gels of viral proteins of cesium chloride-purified DA and DA-259/261 (Met/Lys) virions with or without trypsin treatment prior to cesium chloride gradient purification. Untreated DA-259/261 (Met/Lys) virus (lane B) has an uncleaved VP1 which has the same electrophoretic mobility as VP1 of DA wild-type virus. Trypsin treatment of DA-259/261 (Met/Lys) virus (lane A) cleaves VP1; the cleavage product migrates just above VP2 and has the same mobility as seen following trypsin treatment of DA virus (lane C).

the mutated Iso and Leu were changed back to Arg and Lys respectively was infectious (Figure 3).

Constructs in which Arg was mutated to Met, while residue 261 Lys was maintained as wild-type were infectious (Figure 3). Figure 4, lane A demonstrates that trypsin treatment of virus with the VP1 259 Arg mutation, like wild-type DA virus (Figure 4, lane C) leads to a decrease in the mobility of VP1 compared to untreated virus (Figure 4, lane B). In contrast, constructs in which residue 261 Lys was mutated to Glu while residue 259 Arg was maintained as wild-type were not infectious. These data suggest that the Lys at VP1 residue 261 is the site of trypsin cleavage and is necessary for infectivity. In the case of GDVII virus, the Lys at residue 263 is the only Lys or Arg positioned to yield a size reduction of about 2000 daltons, because there is no Arg aligned with DA 259 Arg; therefore, like its aligned DA residue VP1 261, GDVII VP1 263 is the likely site of trypsin cleavage.

Mutations of the TMEV VP1/2A dipeptide junction affect processing, virus viability and disease phenotype

The VP1/2A processing junction of TMEV has been predicted to be glutamic acid/asparagine (Glu/Asn) through sequence alignment with encephalomyocarditis virus, another member of the cardiovirus genus. We mutated DA and GDVII VP1/2A Glu/Asn to confirm its localization at this junction; we also

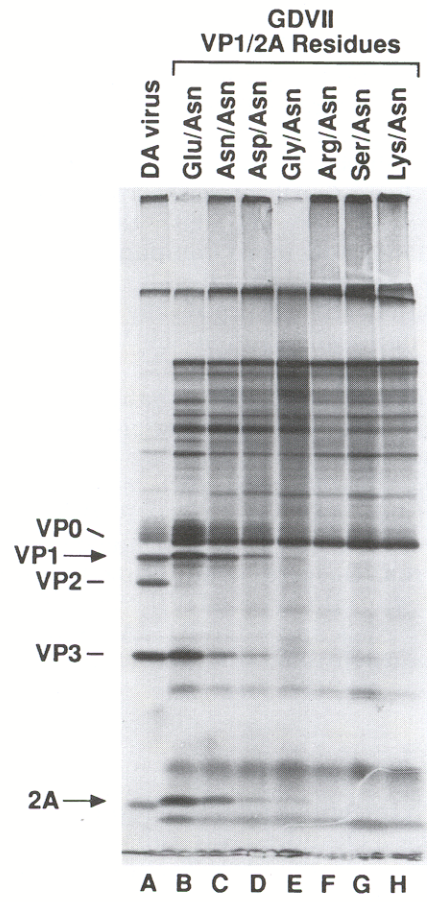

Figure 5 *In vitro* translation of RNA transcripts derived from wild-type pGDVIIFL2 (lane B) or pGDVII VP1/2A junction-mutant clones (lanes C—H). Lane A is a marker lane with [^{35}S] methionine-labelled proteins from an extract of DA-virus infected BHK-21 cells harvested 14 h after infection. The GDVII VP1/2A dipeptide junction sequence is specified on top of lanes B–H.

determined the effects of the mutations on GDVII neurovirulence.

Constructs with mutations of DA VP1/2A Glu/Asn to Ala/Ser, Ala/Asn and Glu/Ser all yielded infectious virus. These data show that amino acid residues with a significant variation in volume and charge can be recognized by the viral protease 3C and processed efficiently at the DA VP1/2A dipeptide junction. We then mutated GDVII VP1 276 Glu at the predicted GDVII VP1/2A Glu/Asn junction to Lys, Asn, Ser, Arg, Asp, or Gly. *In vitro*-derived RNA transcripts from pGD-VP1/2A (Asp/Asn) and pGD-VP1/2A (Asn/Asn) were infectious, although plaque size of the resultant viruses was reduced compared to GDVII wild-type virus. The other clones (with Lys/Asn, Ser/Asn, and Gly/Asn), however, were not infectious. These data suggest that GDVII infectivity is affected by volume changes when GDVII VP1 276 Glu is mutated.

We investigated processing at the VP1/2A junction by *in vitro* translation of *in vitro*-derived tran-

scripts from the wild-type and mutated clones. *In vitro* translation of RNA derived from the non-infectious clones showed an absence (Figure 5, lanes F, G, H) or significant reduction (Figure 5, lane E) in VP1 and 2A relative to the precursors of these proteins. In contrast, the infectious mutated constructs (Figure 5, lanes C, D), processed precursor proteins to VP1 and 2A more efficiently than the non-infectious mutants and more comparable to wild type GDVIIFL2 (Figure 5, lane B). The effect of mutation of Glu/Asn on VP1/2A processing and on infectivity supports its localization at the VP1/2A dipeptide junction.

We questioned whether changes at the GDVII VP1/2A dipeptide junction might change disease phenotype because of a slowing of the kinetics of processing. For this reason we tested whether GD-VP1/2A (Asp/Asn) and GD-VP1/2A (Asn/Asn) viruses were altered in neurovirulence compared to GDVII virus. Despite a reduction in plaque size compared to wild-type GDVII virus, GD-VP1/2A (Asn/Asn) virus and GD-VP1/2A (Asp/Asn) virus were as neurovirulent as GDVII with LD_{50}s of < 3.4 PFU and < 7.2 PFU respectively.

Discussion

Full-length infectious clones of representative members of the TO and GDVII subgroups have been powerful tools in the identification of determinants of TMEV-induced CNS disease. One approach to identifying these determinants is to exchange parts of DA and GDVII infectious clones and to test the disease phenotype of the recombinant viruses. These studies have demonstrated that a major neurovirulence determinant falls within the GDVII capsid protein-encoding region (Calenoff *et al*, 1990; Fu *et al*, 1990b; Pritchard *et al*, 1993; Zhang *et al*, 1993), and that GDVII virus has demyelination determinants not apparent following inoculation with this virus due to the rapid deaths it induces (Fu *et al*, 1990a). To further knowledge regarding the effect of single amino acids on TMEV-induced CNS disease, we used, as described in this paper, site-directed mutagenesis of a full-length infectious DA, GDVII and GDVII/DA chimeric cDNA.

The studies reported here confirm our previous investigations involving mAb escape-mutant viruses that suggested the importance of DA VP1 268 in the late demyelinating disease (Roos *et al*, 1989). This amino acid may affect demyelination by its presence within a neutralization site or because of its proximity to the putative receptor binding site of the cells. Crystallographic studies show that the last ordered VP1 residue on the DA electron density map is 256, so that the location of DA VP1 residues 257–274 cannot be definitely assigned (Luo *et al*, 1987; Grant *et al*, 1992). As part of what may be a mobile region that lies along the rim of the 'pit,' DA VP1 268 would presumably have the potential to influence receptor binding. In addition to DA VP1 268, there are two other amino acids, VP1 101 and VP2 141, that have been shown to influence the late demyelinating disease (Jarousse *et al*, 1994; Zurbriggen *et al*, 1991). Interestingly, these two other amino acids are also either known or predicted to be part of a neutralization site (Pevear *et al*, 1988; Boege *et al*, 1991; Jarousse *et al*, 1994) and to border the 'pit' (Luo *et al*, 1987; Grant *et al*, 1992). Nude mouse studies have shown that VP1 101 mutant virus replicates more slowly than wild-type virus (Zubriggen *et al*, 1991). This finding suggests that the effect of VP1 101 (and, by extrapolation, perhaps VP1 268 and VP2 141) is not mediated solely by its involvement with the immune system; these residues may influence demyelination, at least partly, by interfering with the binding of virus to oligodendrocytes and/or macrophages considering the proximity of these residues to the 'pit'. The mechanisms by which these residues affect the late demyelinating disease may involve both the immune system as well as virus binding to cell receptors.

Our previous study of GDVII/DA recombinant viruses suggested that GDVII has determinants for demyelination which are obscured because of the rapid fatality this virus induces (Fu *et al*, 1990a). We made use of a non-fatal demyelinating GDVII/DA recombinant virus that contained GDVII VP1 in order to test the effect on demyelination of a mutation of GDVII VP1 270, the residue aligned in sequence with DA VP1 268. In contrast to the findings with DA virus, there was no change in the demyelinating activity of GD1B-2C/DAFL3 virus when Val was mutated to Phe. These results suggest that DA VP1 286 and GDVII VP1 270 are not functionally equivalent or that amino acid residues can function differently when present in recombinant viruses than when present in parental viruses, perhaps because of disturbed interactions between the residue and other parts of the genome. The latter explanation is supported by our findings that: GDVII VP1 270 Val to Phe reduces neurovirulence when present in GD1B-2C/DAFL3 virus but not when present in GDVI virus; the presence of asparagine rather than lysine as DA VP2 141, has been shown to attenuate demyelination in a recombinant virus, but has minimal if any effect when present in DA virus (Jarousse *et al*, 1994). These results demonstrate the difficulty of drawing conclusions regarding the pathogenic role of a particular residue with respect to the parental virus based on observations made from investigations using recombinant viruses.

Because DA VP1 268 is part of a neutralization epitope that is disrupted by trypsin cleavage and because demyelination is partially immune-mediated (reviewed by Roos and Casteel, 1992), we were interested in identifying the site of trypsin cleavage and determining the consequences of trypsin cleav-

age on disease. Results of our mutagenesis studies suggest that DA VP1 residue 261 Lys is the site of *in vitro* trypsin cleavage and is critical for virus viability. The effect on virus viability may be related to an interference with binding to the cell receptor, since DA VP1 Lys is predicted to lie along the rim of the 'pit' (Luo *et al*, 1987; Grant *et al*, 1992); *in vitro* studies of the picornaviruses foot-and-mouth disease virus and coxsackie A-9 virus have demonstrated that trypsin treatment alters virus binding to cell receptors (Wild and Brown, 1967; Fox *et al*, 1989; Roivainen *et al*, 1991). Since we were unable to produce infectious virus that was resistant to trypsin cleavage, we could not determine the effect of such a mutation on disease phenotype.

The present study also investigated the effect of mutations at the VP1/2A dipeptide junction. The TMEV VP1/2A junction, as well as a number of other junctions of the polyprotein, are cleaved by the virally-encoded protease 3C (Roos *et al*, 1989; Lawson and Semler, 1990; Palmenberg, 1990). Studies of several picornaviruses have demonstrated that cleavage by the viral proteinases is controlled by the sequence of the pair of amino acids at the cleavage site as well as surrounding residues, and the spatial conformation of the capsid proteins (Nicklin *et al*, 1987; Parks and Palmenberg, 1987; Ypma-Wong and Semler, 1987; Lee and Wimmer, 1988; Ypma-Wong *et al*, 1988; Parks *et al*, 1989; Hellen *et al*, 1992). We tested whether mutations of the predicted TMEV VP1/2A dipeptide junction, Glu/Asn, affect processing and the virus' disease phenotype. Mutations of DA VP1/2A Glu/Asn to Ala/Ser, Ala/Asn and Glu/Ser all yielded infectious virus. These data show that DA clones can tolerate significant variations in size and charge at this dipeptide junction and remain infectious. In contrast, clones with a mutation of the GDVII VP1/2A junction to Asp/Asn and Asn/Asn were infectious, while clones with a change to Gly/Asn, Arg/Asn, Lys/Asn, and Ser/Asn were not. Thus, GDVII clones involving mutations of VP1 276 Glu are less tolerant of changes in size than in charge.

The efficiency of processing of *in vitro*-translated lysates correlated well with infectivity of the GDVII VP1/2A mutated clones. Clones that had little or no processing at the VP1/2A junction were not infectious. Investigations of poliovirus have also demonstrated a correlation between the kinetics of cleavage at processed junctions and infectivity (Blair and Semler, 1991; Hellen *et al*, 1992). The demonstrated effect of mutation of GDVII VP1 276 on VP1/2A processing (and infectivity), as well as the alignment with encephalomyocarditis virus, support the localization of Glu/Asn at the VP1/2A processing junction.

The two infectious GDVII VP1/2A mutated clones produced small plaque viruses following transfection, while GDVII is a large plaque virus. These mutant viruses were not attenuated with respect to neurovirulence. Although small plaque size has been associated with attenuation, this correlation is imperfect (Calenoff *et al*, 1990; Fu *et al*, 1990) as is true in this case. It is possible that these viruses are impaired for growth in BHK-21 cells, resulting in small plaque size, but not impaired in neurons.

This study demonstrates the importance of the VP1 carboxyl region in TMEV-induced disease and in virus infectivity. It also emphasizes the role in disease of residues that border the putative receptor binding site and are included within a neutralization epitope. Future studies involving the inoculation of mutant viruses in Nude or immunocompromised mice and comparing the *in vitro* binding of wild-type and mutant viruses to oligodendrocytes may help clarify the mechanisms involved.

Materials and methods

Cells

L929 cells (mouse fibroblasts) or BHK-21 cells (baby hamster kidney cells) were used for plaque assays and transfections, and BHK-21 cells were used for the growth of stock virus.

In vitro *transcription and transfection*

Transcription templates were linearized with Xba I (Boehringer Mannheim, Indianapolis, IN), which cuts 3' to the end of the TMEV genome, then transcribed in a T7 RNA polymerase reaction (Pharmacia, Piscataway, NJ). The resultant RNAs were transfected into L cells or BHK-21 cells as previously described (Roos *et al*, 1989). The monolayer of the transfected cells was scraped after it exhibited maximal cytopathic effect. The resultant virus was then plaque purified twice in L cells or BHK-21 cells, and passed twice in BHK-21 cells to produce a sufficient amount of stock virus.

Animal inoculations

Weanling (4-6 week old) SJL/J mice (Jackson Laboratory, Bar Harbor, ME) were inoculated intracerebrally with 0.03 ml of undiluted virus or 10-fold dilutions of virus. Late demyelinating disease was evaluated at 6 weeks to 3 months. The pathological features that were evaluated in this late disease consisted of inflammation and demyelination of the white matter. Mice were sacrificed, and the brains and spinal cords were removed, formalin-fixed and paraffin-embedded. Sections of the brains and spinal cords were stained with hematoxylin and eosin. Neurovirulence was evaluated by recording mouse deaths for 4 weeks post-inoculation to arrive at a maximal or minimal 50% lethal dose (LD_{50}) (Karber, 1931).

Construction of plasmids

The templates used for mutagenesis were generally full-length, infectious cDNA clones generated from DA and GDVII, pDAFL3 and pGDVIIFL2 respective-

ly (Roos *et al*, 1989; Fu *et al.*, 1990). pGD1B-2C/DAFL3 recombinant virus (Fu *et al*, 1990b), which contains the coding region for GDVII 1B (VP2) through 2C substituted in the DA genome, was also used for mutagenesis.

Mutagenesis reactions were generally performed by a three-reaction, recombinant PCR method (Higuchi, 1990). The primary PCR mutagenesis reactions, the 'left' and 'right,' contained in a 99.5 µl volume: 10 to 100 ng of plasmid template, 100 pmole of a mutagenesis oligonucleotide (Operon, Alameda, CA), 100 pmole of either an upstream or downstream outside primer of complementary sense to the mutagenesis primer used, 0.2 mM of each dNTP, 50 mM KCl, 10 mM Tris hydrochloride (pH 8.3), 1.5 mM MgCl2, and .001% gelatin. After a 2 min 'hot start' at 95°, 0.5 µl (2.5U) Taq polymerase (Perkin-Elmer, Norwalk, CT) was added beneath a layer of paraffin oil. The mixture was then cycled 35 times (94°C for 1 min, 55°C for 1 min, and 72°C for 2 min) followed by one cycle of 94°C 1 min, 55°C 1 min, and 72°C 10 min. The 'left' and 'right' reaction products were GeneCleaned (Bio 101, La Jolla, CA) from the gel after electrophoresis. The third PCR reaction which filled in and amplified the heteroduplexes was performed as above except that: 10 ng of each primary reaction product was substituted for the original template; also 100 pmol of both outside primers were added. The final product was cut from an agarose gel after electrophoresis, GeneCleaned (Bio 101), and if necessary, used for further cloning. Mutagenesis of the VP1/2A junction did not involve the above mentioned mutagenesis scheme, but employed ligation of annealed, degenerate oligonucleotide pairs.

The following clones were constructed as described in detail elsewhere (Senkowski, 1994): pDA-VP1-268 which changes DA VP1 residue 268 from valine (Val) to phenylalanine (Phe); pGD1B-2C (VP1 270)/DAFL3 which changes GD1B-2C/DAFL3 VP1 residue 270 from Val to Phe; pGD-VP1-270 which changes GDVII VP1 residue 270 from Val to PHe; pDA-VP1-259/261 (Iso/Leu) which changes DA VP1 259 and 261 from arginine (Arg) and lysine (Lys) to isoleucine (Iso) and leucine (Leu); pDA-VP1-259/261 (Met/Gln), pDA-VP1-259/261 (Arg/Gln), pDA-VP1-259/261 (Arg/Lys) and pDA-VP1-259/261 (Met/Lys) which change DA-VP1-259/261 (Iso/Leu) VP1 residues 259 and 261 to methionine (Met)/glutamine (Gln), Arg/Gln, Arg/Lys and Met/Lys respectively; pDA-VP1/2A (Ala/Asn), pDA-VP1/2A (Ala/Ser), and pDA-VP1/2A (Glu/Ser) which change DA VP1 residue 274 and DA 2A residue 1 from glutamic acid (Glu) and asparagine (Asn) to alanine (Ala)/Asn, Ala/serine (Ser), and Glu/Ser respectively; pGDVII-VP1/2A (Asp/Asn), pGDVII-VP1/2A (Asn/Asn), pGDVII-VP1/2A (Gly/Asn), pGDVII-VP1/2A (Lys/Asn), pGDVII-VP1/2A (Ser/Asn), and pGDVII-VP1/2A (Arg/Asn) which change GDVII VP1 residue 276 and GDVII 2A residue 1 from Glu and Asn to aspartic acid (Asp)/Asn, Asn/Asn, glycine (Gly)/Asn, Lys/Asn, Ser/Asn, and Arg/Asn respectively.

Cesium chloride gradient purification and trypsin treatment of virus

TMEV was purified using cesium chloride equilibrium gradient centrifugation as previously described (Roos *et al*, 1982). In some cases virus was treated with trypsin (Sigma, St Louis, MO) at a final concentration of 0.5 mg ml^{-1} (37°C for 10 min) prior to ultracentrifugation, as previously published (Nitayaphan *et al*, 1985a). After treatment, a trypsin inhibitor (Sigma) was added at a final concentration of 0.5 mg ml^{-1}. Following purification an aliquot of the virus was subjected to electrophoresis on 12.5% polyacylamide gels containing sodium dodecyl sulfate (SDS) and stained with Coomassie blue.

Western blots

Western blots were run to delineate whether virus had an epitope for a particular monoclonal antibody (mAb). Stocks of virus were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose paper by electroelution and processed for immunoblotting as described (Nitayaphan *et al*, 1985b). The first antibody was one of two TMEV mAbs that has been previously described (Nitayaphan *et al*, 1985b) and the second overlay was peroxidase-conjugated sheep antimouse IgG serum. The immunoblot was developed with diaminobenzidine and hydrogen peroxide.

In vitro *translations*

Fifty to 200 ng of *in vitro*-derived RNA transcripts were translated in rabbit reticulocyte lysate at 30°C for 3 h as described previously (Roos *et al*, 1989). Reactions were terminated with either Laemmli sample buffer or by incubation with 0.5 mg ml^{-1} RNase and 0.005 mg ml^{-1} cyclohexamide at 30°C for 20 min. The translated lysates were subjected to SDS-polyacrylamide gel electrophoresis as described above. The gels were dried and exposed to XAR film for autoradiography.

DNA and RNA sequencing

DNA sequencing was performed by a dideoxynucleotide chain termination method (Sanger *et al*, 1977) with the 'fmol' dsDNA cycle sequencing system (Promega, Madison, WI). Reactions were carried out according to the instructions of the manufacturer.

RNA from viruses that had undergone site-directed mutagenesis were sequenced by a reverse transcriptase PCR method with removal of primers from the reverse transcription step using PrimeErase columns (Stratagene, La Jolla, CA). Total RNA, including viral RNA, was isolated by addition of 100 µl of tissue-culture grown stock of virus to 900 µl of RNAzol (Cinna/Biotecx, Houston, TX) with

additional steps performed according to the manufacturer's instructions. The DNA that resulted from reverse transcription PCR was sequenced using the 'dsDNA Cycle Sequencing System' (Gibco BRL, Gaithersburg, MD).

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