Pathogenesis of Early and Late Disease in Mice Infected with Theiler's Virus, Using Intratypic Recombinant GDVII/DA Viruses

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Intratypic recombinant Theiler's viruses prepared between GDVII and DA strains were used to identify genomic sequences important in neurovirulence, virus persistence, and demyelination and to clarify the mechanisms involved in disease induction. The coding region between 1B and 2C of the highly virulent GDVII strain contains a determinant partly responsible for neurovirulence (early paralysis and death) which correlates with elevated levels of infectious virus and the presence of virus antigen within neurons of the brain stem and gray matter of the spinal cord. Both the GDVII and the DA strains of virus contain genetic determinants for late demyelination in spinal cord. However, quantitative analysis of demyelination produced by recombinant GDVII/DA viruses suggest that multiple gene segments influence the number and extent of demyelinating lesions.

Theiler's murine encephalomyelitis viruses (TMEV) are picornaviruses that naturally induce enteric and neurologic disease of mice (19, 27). TMEV strains can be divided into two subgroups on the basis of neurovirulence (8). GDVII and other members of the GDVII subgroup produce acute fatal polioencephalomyelitis (7, 8). In contrast, Daniels (DA) strain (2) and other members of Theiler's original (TO) subgroup (6) persist in the central nervous system (CNS) and result in chronic inflammatory demyelinating disease of the spinal cord throughout the life of the animal. Infectious cDNA clones of DA and GDVII strains of TMEV have been produced; chimeric cDNAs and intratypic recombinant viruses have been generated to identify which parts of the genome are important in neurovirulence (acute fatal encephalitis), virus persistence, and chronic CNS demyelination (1, 4, 5, 9, 10, 23, 25, 26). Our previous studies (4, 5) have indicated that a genomic segment from the GDVII 1B to 2C coding region contains a major determinant for neurovirulence, the addition of the GDVII genome 5' to the 1B to 2C coding segment also contributes to neurovirulence, and strains from both TMEV subgroups encode determinants for demyelination.

The major goal of the present study was to further delineate the molecular basis of the biological activities of the prototypic virus strains DA and GDVII. We used detailed morphologic quantitative studies to characterize the neuropathology and immunocytochemistry to detect virus antigen in the spinal cord and brains of mice infected intracerebrally with intratypic recombinant GDVII/DA viruses. In addition, the growth cycle of one intratypic recombinant virus was analyzed in weanling mouse brain and in vitro in BHK-21 cells to determine the kinetics of viral growth. The experiments suggested that a critical factor in determining neurovirulence of GDVII virus subgroup was the rapid and extensive infection of vital neuronal structures in the brain stem and spinal cord gray matter. Even though all TMEV strains encode a genetic determinant for demyelination and virus persistence, quantitative studies demonstrated that substituting the 2C to 3C coding region or the 5'

to 1B coding region of GDVII within the DA genome resulted in dramatic reduction in the extent of demyelination despite continuing presence of virus antigen and infectious virus in the spinal cord. These results indicate that multiple genetic determinants can affect the late demyelinating disease.

MATERIALS AND METHODS

Virus. Viruses obtained from transfection of in vitro-derived transcripts of infectious DA or GDVII cDNA clones are designated DAFL3 and GDVIIIFL2 respectively and have been fully described (5, 23). A series of chimeric cDNAs were constructed in which a segment of an infectious DA cDNA clone or a segment of an infectious GDVII cDNA clone was substituted by the corresponding segment of the other strain (23) (Fig. 1). Details regarding the construction of these chimeric cDNAs have been published (23). The chimeric cDNAs are named by listing the upstream and downstream boundaries of the substituted segment derived from the parental clone that makes up the minority of the chimera, which is followed by a slash and then the name of the other parental clone. The chimeric cDNAs were transcribed and the transcripts transfected into L929 cells to produce recombinant viruses as previously described (23).

Virus growth cycle in vitro. BHK-21 cells in 35-mm dishes were adsorbed in duplicate for 1 h with DAFL3, GDVIIIFL2, or GD1B-2C/DAFL3 viruses at a multiplicity of infection of approximately 10. The monolayers were washed and scraped at 2-h intervals from 1 to 10 h after infection. Cells and supernatants were assayed for infectivity on a plaque assay on L cells, as previously described (22).

Virus growth cycle in weanling mouse CNS. Various amounts of DAFL3, GDVIIIFL2, and GD1B-2C/DAFL3 viruses were separately inoculated intracerebrally into groups of 19 SJL/J mice. Two animals from each group were sacrificed each day until day 10 or until none remained. The brains and spinal cords were made into a 10% homogenate in Hank's buffered saline solution and infectivity assayed as described above.

Animals and experimental protocol. SJL/J mice (4 to 6 weeks of age) from the Jackson Laboratory (Bar Harbor,

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FIG. 1. TMEV chimeric intratypic cDNAs and their phenotypes as related to neurovirulence, demyelination, and virus persistence. Neurovirulence was graded as follows: -, attenuated; +, somewhat neurovirulent; ++, highly neurovirulent. Demyelination was graded as follows: +++++, demyelination score of >35; +++, demyelination score of 25 to 34.9; ++, demyelination score of 15 to 24.9; +, demyelination score of <15; 0, no demyelination. Some of the data regarding neurovirulence and demyelination have been previously published (4, 5).

Maine) were inoculated intracerebrally with various doses of TMEV or GDVII/DA recombinant viruses in 30 µl of vehicle. Animals were monitored daily for the presence of paralysis or death until day 7, 14, or 45 after infection.

Assay for infectious virus in spinal cords of inoculated mice. Forty-five days after inoculation with the different recombinant viruses, mice were sacrificed and the spinal cords were removed aseptically and frozen. At a later date, a 10% homogenate of the cord was prepared in Hank's buffered saline solution and assayed for infectious virus as described above.

Immunocytochemistry to detect virus antigen. Immunocytochemistry was performed on spinal cords or brains from infected mice which were either perfused with fixative or sacrificed fresh for frozen sections. For fixative-perfused mice, spinal cord sections stored in 0.1 M phosphate buffer were rinsed in 0.1 M Tris buffer with 25 mM hydroxylamine (pH 7.4), treated with 10% dimethyl sulfoxide in the same buffer for 1 h, and quick-frozen in isopentane chilled in liquid nitrogen. Ten-micrometer cryostat sections were cut and transferred to gelatin-coated glass slides. Frozen spinal cord or brain sections were reduced with 1% sodium borohydride in 0.1 M Tris buffer with 25 mM hydroxylamine (pH 7.4) at 4°C and refixed with 95% alcohol-5% glacial acetic acid. For animals which did not undergo in vivo fixation, spinal cords and brains were quick-frozen, sectioned, and then fixed for 20 min in acetone (-20°C). Sections were immunostained with a polyclonal antiserum to purified TMEV strain DA virions (18) by the avidin-biotin immunoperoxidase technique (Vector Laboratories, Burlingame, Calif.). Slides were developed with a solution of Hanker-Yates reagent (*p*-phenylenediamine-procatechol [Polysciences]) and counter stained with hematoxylin.

Quantitative pathologic analysis in spinal cords (45 days following infection). Mice were perfused with Trump's fixative (phosphate-buffered 4% formaldehyde containing 1% glutaraldehyde [pH 7.4]), and the spinal cords were pro-

cessed to provide 2-µm-thick glycol methacrylate-embedded sections. A detailed morphologic analysis was performed on each of 10 to 15 coronal spinal cord sections from each animal. A pathologic score based on gray matter inflammation, meningeal inflammation, and demyelination was obtained for each animal as previously described (20). Each quadrant from every coronal section from each mouse was scored for the presence or absence of pathologic abnormalities. The score was expressed as a percent over the total number of quadrants examined. The maximum pathologic score, 100, indicated the presence of inflammation or demyelination or both in every quadrant of all spinal cord sections of one mouse. Student's *t* test was used to determine statistical significance of differences in pathologic scores between groups of animals that were inoculated with various GDVII/DA recombinant viruses.

RESULTS

Studies of TMEV neurovirulence. Mice were infected with two parental strains (GDVIIIFL2 and DAFL3 viruses) and GDVII/DA intratypic recombinant viruses and monitored for paralysis or death (Fig. 1). We operationally defined neurovirulence as death of a weanling mouse within 1 month of intracerebral inoculation. Animals infected with GDVIIIFL2 virus demonstrated consistently severe paralysis and early death within the first 2 to 4 weeks of infection, even when these mice were inoculated with as little as 5 to 10 PFU of virus. In contrast, none of 18 mice infected with DAFL3 virus became paralyzed or died within the first 4 weeks of infection with 3×10^4 PFU of input virus. Of the mice infected with GDVII/DA recombinant viruses, none inoculated with GD5'-1B/DAFL3 (*n* = 15), GDNC/DAFL3 (*n* = 9), GD2C-3C/DAFL3 (*n* = 15), or GD3C-3'/DAFL3 (*n* = 15) developed clinical abnormalities or death within a month following inoculation. In contrast, prominent neurologic abnormalities were noted in mice infected with recombinant

FIG. 2. Growth cycle of DAFL3, GDVIIIFL2, and GD1B-2C/DAFL3 viruses in BHK-21 cells. Details regarding methods are given in the text. The individual datum points represent the mean of two separate titrations, and a bar shows the standard error of the mean. The bar is not visible for some datum points because of a small standard error of the mean or because, in one case, only one datum point was available (GDVIIIFL2 virus at 1 h postinfection).

GD1B-2C/DAFL3 virus. Five of six mice infected with GD1B-2C/DAFL3 virus (3×10^4 PFU) died on average on day 12. The one surviving mouse scheduled for sacrifice on day 45 showed severe quadriplegia. Four of five mice infected with GD1B-2C/DAFL3 virus (7.5×10^3 PFU) died on average on day 17. None of five mice infected with GD1B-2C/DAFL3 virus (7.5×10^2 PFU) died, but four of the five developed paralysis that was apparent at the time of sacrifice on day 45. In contrast, none of five mice infected with GD1B-2C/DAFL3 virus (75 PFU) showed paralysis or death.

Investigations of mechanisms for neurovirulence. (i) Virus growth in vitro. In an effort to begin to clarify the reason(s) for neurovirulence of the GDVII subgroup of viruses, we chose to more extensively study two neurovirulent viruses, GDVIIIFL2 and GD1B-2C/DAFL3, and compare their properties with the nonneurovirulent parental strain, DAFL3 virus. The growth cycle after inoculation of these viruses in BHK-21 cells showed that by 9 h after infection, GDVIIIFL2 and GD1B-2C/DAFL3 viruses had reached levels that were similar to each other and approximately 100-fold greater than DAFL3 virus (Fig. 2). Similar results comparing DA and GDVII strains have been previously reported (3).

(ii) Virus growth in weanling mouse CNS. To make these studies more relevant to investigations of neurovirulence, the growth cycles of these three viruses in weanling mouse brain and spinal cord were studied. Weanling mice were infected and assayed daily for levels of infectious virus (Fig. 3A and B). We chose an input dosage of GDVIIIFL2 virus (9.0×10^3 PFU) that always kills mice and a dosage of DAFL3 virus (2.4×10^5 PFU) that never kills weanling mice. For GD1B-2C/DAFL3 virus, one dosage that usually kills (4.8×10^5 PFU) and one dosage that usually does not kill (4.8×10^2 PFU) were chosen.

GDVIIIFL2 virus, the most neurovirulent of the three viruses tested, had the most efficient growth in weanling mouse CNS, despite an initial inoculation of only 9.0×10^3 PFU. Mice inoculated with the usually lethal dose of GD1B-2C/DAFL3 virus had substantially higher titers of virus in CNS on days 1 through 7 compared with those in mice inoculated with a dose of GD1B-2C/DAFL3 virus that is not

FIG. 3. Growth cycle of viruses following intracerebral inoculation of weanling mice. Details regarding methods are given in the text. (A) DAFL3 (circle, individual CNS homogenate titers). Solid line connects average titer and GDVIIIFL2 (square, individual CNS homogenate titers). Dashed line connects average titer viruses. Note that only one animal was sacrificed at day 7 and day 10 for GDVIIIFL2 and DAFL3 viruses, respectively. (B) GD1B-2C/DAFL3 virus inoculated at high concentration with a 10^{-1} dilution of the stock (triangle, individual CNS homogenate titers). Solid line connects average titer and at a low concentration with a 10^{-4} dilution (circle with plus sign, individual CNS homogenate titers). Dashed line connects average titer).

usually lethal. The levels of virus in the CNS following inoculation with the higher input of GD1B-2C/DAFL3 virus were comparable to that seen following inoculation with DAFL3 virus for the first 5 days following infection. However, by day 6, the titer of virus in the CNS of mice inoculated with DAFL3 virus tended to subside while the titer in brains of mice inoculated with the usually lethal dose of GD1B-2C/DAFL3 virus remained high. Therefore, mice inoculated with GD1B-2C/DAFL3 virus had 10 to 1,000 times more virus from day 6 through day 8 (the last day tested with the high dose of GD1B-2C/DAFL3 virus) than those inoculated with DAFL3 virus. Since animals inoculated with the higher dose of GD1B-2C/DAFL3 virus usually died after day 7 postinoculation, death may have been associated with the higher levels of virus that persisted in the

TABLE 1. Virus antigen in CNS following acute infection with recombinant GDVII/DA Theiler's viruses (day 7)^a

Virus	Virus antigen								
	Cerebellum	Brain stem	Cortex	Hippocampus	Thalamus	Corpus callosum	Meninges	Spinal cord	
								Gray matter	White matter
DAFL3	0	+1	+2	+1	+2	0	0	+1	+1
GDVIIIFL2	0	+4	+1	+1	+2	0	0	+4	0
GD1B-2C/DAFL3	0	+3	+3	+2	+3	0	0	+4	0
GD5'-1B/DAFL3	0	0	+1	+1	+2	0	0	0	0
GD2C-3C/DAFL3	0	0	+1	+1	+2	0	0	0	0
GD3C-3'/DAFL3	0	0	0	+1	+2	0	0	0	0

^a Qualitative analysis of number of virus antigen-positive neurons and glial cells (spinal cord white matter) per 25× objective by using immunoperoxidase technique on frozen sections. Three mice were infected with each of the viruses. 0, none; +1, <5 cells; +2, 5 to 10 cells; +3, 10 to 20 cells; +4, >20 cells.

CNS and/or with the presence of virus in particular regions of brain (see below).

(iii) **Localization of virus antigen in the CNS during acute infection.** To further delineate the reasons for early death and severe paralysis in mice infected with GDVIIFL2 or GD1B-2C/DAFL3 virus compared with mice infected with DAFL3

and other recombinant viruses, immunocytochemistry was performed to localize virus antigen in the CNS on day 7 and day 14 following virus infection (Table 1; Fig. 4). On day 7, a difference in the distribution of virus antigen between mice infected with neurovirulent versus nonneurovirulent recombinant viruses was observed. In mice infected with more

FIG. 4. Composite of virus antigen detected by immunoperoxidase technique. (A) Longitudinal spinal cord section from a mouse infected with GDVIIFL2 virus showing multiple antigen-positive cells in the gray matter (g) of the spinal cord. Note relative absence of antigen-positive cells in white matter (w). (B) Localization of virus antigen in brain stem (bs) but not cerebellum (c) in a mouse infected with GDVIIFL2 virus. (C) Prominent virus antigen expression in neurons of the pyramidal (p) layer of the hippocampus but not in dentate (d) in a mouse infected with recombinant GD1B-2C/DAFL3 virus. (D) Virus antigen in the cerebral cortex of a mouse infected with GD1B-2C/DAFL3 virus. All sections were counter-stained with hematoxylin to identify the morphology and detect inflammatory infiltrates. Magnifications, ×127 (A, B, and C) and ×740 (D).

TABLE 2. Pathologic scores in spinal cords of SJL/J mice infected with GDVII/DA recombinant viruses

Virus	Titer (PFU/ml)	No. of mice	% Positive/total quadrants examined (mean $\pm$ SD)		
			Gray matter inflammation	Meningeal inflammation	Demyelination
DAFL3	1×10^6	10	0.2 ± 0.6	32.5 ± 11.6	45.9 ± 13.0
DAFL3	1×10^5	4	1.0 ± 1.1	33.6 ± 7.6	30.8 ± 4.2
DAFL3	1×10^4	4	0.5 ± 1.0	19.0 ± 10.4	15.5 ± 6.5
GD1B-2C/DAFL3	2.5×10^4	5	0.8 ± 1.1	35.1 ± 14.5	35.5 ± 17.4
GD1B-2C/DAFL3	2.5×10^3	6	1.8 ± 3.0	25.3 ± 23.7	21.4 ± 21.9
GD5'-1B/DAFL3	1.6×10^5 or 1.0×10^6	12	0.1 ± 0.6	2.3 ± 5.7	3.5 ± 7.2^a
GDNC/DAFL3	1.0×10^6	4	0.0 ± 0.0	29.6 ± 6.8	27.2 ± 8.4^b
GDNC/DAFL3	5.0 ± 10^4	5	0.0 ± 0.0	1.3 ± 3.9	2.3 ± 5.1
GD2C-3C/DAFL3	1.0×10^6	7	0.0 ± 0.0	1.1 ± 2.2	0.8 ± 1.4^a
GD3C-3'/DAFL3	1.0×10^6	7	0.3 ± 0.8	27.0 ± 20.6	29.9 ± 16.8
GD3C-3'/DAFL3	2.8×10^7	5	0.0 ± 0.0	27.8 ± 7.7	40.1 ± 17.0

^a $P = 0.0001$ (Student's *t* test) compared with DAFL3 (10^6).^b $P = 0.0218$ (Student's *t* test) compared with DAFL3 (10^6).

neurovirulent GDVIIIFL2 or GD1B-2C/DAFL3 viruses, an increase in the number of infected neurons was observed primarily in the brain stem, thalamus and gray matter of the spinal cord. Virus antigen-positive neurons were in intimate association with dense mononuclear infiltrates and necrosis. Antigen-positive cells were also observed in the cerebral cortex, but more so in mice infected with GD1B-2C/DAFL3 virus than in mice infected with GDVIIIFL2 virus; this result was surprising, since brains from GDVIIIFL2 virus-inoculated mice tended to have more infectious virus than did those from mice inoculated with GD1B-2C/DAFL3 virus, and remains unexplained. Fewer virus antigen-positive neurons were observed in cortex, hippocampus, and thalamus in mice infected with nonneurovirulent viruses (i.e., DAFL3, GD5'-1B/DAFL3, GD2C-3C/DAFL3, and GD3C-3'/DAFL3). On day 7 after infection, antigen-positive cells were only detected in spinal cord white matter in mice infected with DAFL3 virus. No virus antigen-positive cells were observed in the cerebellum or corpus callosum in any of the infected mice. Similar pattern of results were obtained with immunohistochemical studies performed 14 days following infection.

Investigations of mechanisms for demyelination and virus persistence. (i) **Quantitative analysis of demyelination in the spinal cords of mice chronically infected with recombinant GDVII/DA viruses.** To determine which virus segments of the DA or GDVII genome correlated with CNS demyelination, SJL/J mice were infected with GDVII/DA recombinant virus, and spinal cords were analyzed 45 days following infection by using plastic-embedded sections. For statistical comparisons, mice were inoculated with similar amounts of virus. As reported previously (4), mice infected with DAFL3 virus or with any of the recombinant GDVII/DA viruses developed chronic demyelination and meningeal inflammation.

Major differences were noted in the number and extent of demyelinating lesions in the spinal cords of mice infected with various recombinant viruses. Mice infected with GD1B-2C/DAFL3 or GD3C-3'/DAFL3 viruses had extensive spinal cord demyelination similar to that in mice infected with DAFL3 virus (Table 2; Fig. 1). Mice infected with GD1B-2C/DAFL3 (2.5×10^4 PFU/ml) demonstrated that 36% of 300 spinal cord quadrants showed demyelination, whereas mice infected with GD3C-3'/DAFL3 (10^6 PFU/ml) demonstrated that 30% of 420 spinal cord quadrants showed demyelination. In contrast, mice infected with GD5'-1B/DAFL3 virus (10^5 or 10^6 PFU/ml) had an average of 3.5% of

180 spinal cord quadrants with demyelination (Student *t* test, $P = 0.0001$ compared with DAFL3). A statistically decreased pathologic score for demyelination in spinal cord was also observed in mice infected with GDNC/DAFL3 virus (27.2 ± 8.4) compared with that in mice infected with DAFL3 virus (45.9 ± 13.0 ; $P = 0.0218$). Of interest is the fact that mice infected with highly neurovirulent GD1B-2C/DAFL3 virus showed more prominent CNS demyelination (Table 2), even though lower titers of input virus (2.5×10^4 or 2.5×10^3 PFU/ml) were inoculated in order to prevent early paralysis and death and to permit the development of the late chronic demyelination.

To determine if mice infected with GD1B-2C/DAFL3 virus at lower input virus had a greater propensity for CNS demyelination than mice infected with DAFL3 virus, the effects of various virus input dilutions in SJL/J mice infected with DAFL3 or GD1B-2C/DAFL3 viruses were compared (Table 2). Pathologic scores for demyelination in spinal cord gradually decreased as the amount of input DAFL3 virus was lowered from 3×10^4 to 3×10^2 PFU. However, all mice inoculated with the lower amounts of DAFL3 virus showed demyelination of at least 15% of spinal cord quadrants. Comparison between mice infected with GD1B-2C/DAFL3 and DAFL3 viruses at approximately 3×10^2 PFU demonstrated more demyelinated lesions in mice infected with GD1B-2C/DAFL3 virus; however, this difference was not statistically significant. No major differences in the degree of destruction of anterior horn cells was observed 45 days after infection with any of the recombinant GDVII/DA viruses, emphasizing the specificity of the neuropathology to spinal cord white matter during chronic infection.

(ii) **Persistence of virus antigen and infectious virus in spinal cord during chronic infection.** To determine if the presence of demyelination correlated with persistence of virus antigen in the spinal cord, immunocytochemistry was performed to localize virus antigen in the spinal cord 45 days following virus infection (Fig. 1 and 5). Virus antigen was demonstrated in the spinal cord white matter of mice infected with each of the GDVII/DA recombinant viruses as well as in mice infected with DAFL3. In general there was a correlation between the number of virus antigen-positive cells with the degree of demyelination. Most lesions had one to five virus antigen-positive cells in spinal cord white matter. The greatest number of virus antigen-positive cells was observed in the spinal cord white matter of mice infected with the highly neurovirulent GD1B-2C/DAFL3 virus. Infectious vi-

FIG. 5. Primary demyelination and virus antigen expression in the spinal cord of mice infected with various recombinant GDVII/DA viruses (day 45). (A) Large area of demyelination in association with perivascular and meningeal inflammatory infiltrates in an SJL mouse infected with GD1B-2C/DAFL3 virus (750 PFU) (glycol methacrylate-embedded section). (B) Frozen section of spinal cord obtained from the same animal as in panel A showing virus antigen-positive cell (arrow) in the white matter (immunoperoxidase staining). (C) Smaller area of demyelination associated with minimal inflammatory infiltrates in a mouse infected with GD2C-3C/DAFL3 virus (3×10^4 PFU). The number of demyelinated lesions in mice infected with this recombinant virus was much less compared with those in mice infected with DAFL3 virus (see Table 2) (glycol methacrylate-embedded section). (D) Frozen section of spinal cord obtained from the same mouse as in panel C showing virus antigen-positive cells (arrows) (immunoperoxidase staining counterstained with hematoxylin). Magnification, $\times 870$.

rus was recovered by plaque assay from spinal cord homogenates of at least one mouse from the groups infected with DAFL3 virus and each of the recombinant viruses. Since all mice infected with GDVIIFL2 died prior to analysis on day 45, it was not possible to determine if virus persisted in these mice.

DISCUSSION

Theiler's virus infection provides an excellent model for the characterization of viral genes responsible for neurovirulence, viral persistence, and demyelination. The two prototypic strains of virus, GDVII and DA, cause very different diseases when injected intracerebrally into susceptible mice, thus providing the opportunity to identify determinants for the various phenotypes. In spite of the marked biologic differences of the two TMEV subgroups, all Theiler's virus strains are closely related at the molecular level (13, 16, 17). The genomes of strains of DA, BeAN, and GDVII are more than 90% identical, whereas their proteins are 95% identical at the predicted amino acid level. The highest level of nucleotide identity is in the 5' and 3' noncoding regions of the genome. In contrast, the major amino acid differences occur in the structural and leader proteins of the viruses encoded by the P1 and L regions of the genome (Fig. 1). This level of identity, however, is too low to permit the mapping of neurovirulence, viral persistence, or demyelination solely on nucleotide sequence differences.

In an effort to further define the genes important to the contrasting phenotypes, we (4, 5) and others (1, 10, 25) have developed GDVII/DA chimeric infectious cDNAs to produce intratypic recombinant viruses. We have published data that neurovirulence is of multigenic origin and that one or more determinants map within the GDVII 1B-2C coding region (4). This identifies a fragment that runs from GDVII nucleotide 1965 in the VP2(1B) coding area to GDVII nucleotide 4843 in the 2C coding area, coding for 80 amino acid differences when compared with DA. Our present studies comparing the growth cycle and location of virus antigen in GD1B-2C/DAFL3 virus with GDVIIFL2 and DAFL3 viruses suggest that the development of fulminant acute encephalomyelitis with subsequent death relates to the ability of GDVII and the other neurovirulent recombinant virus to grow efficiently within the CNS and to infect neurons in the brain stem and gray matter of the spinal cord. Infection of the vital structures controlling respiration, cardiovascular function, and motor activity presumably results in early paralysis and death within 4 weeks of infection.

However, it is less clear which of the genes are important for virus persistence and the demyelination observed after infection with the TO subgroup of TMEV. We have previously reported that both the GDVII and DA strains contain genetic determinants for demyelinating activity (4). We reached this conclusion because intratypic recombinant studies demonstrated that virus that contained a replacement of any part of the DA genome with the GDVII genome still caused demyelination in infected survivors. It was presumed that no demyelination occurs following infection with the more neurovirulent viruses because they produce lethal neuronal disease before demyelination can occur. A key recombinant virus in our study was GD1B-2C/DAFL3, which was only partly neurovirulent but produced demyelination in survivors. We have recently generated an additional recombinant virus, GD1B-2A/DAFL3, which also produces demyelination in survivors (data not shown). The presence of demyelinating determinants in both DA and

GDVII viruses suggests that the use of GDVII/DA recombinant viruses in the precise identification of these determinants is difficult.

The present study suggests that determinants for virus persistence are also present in both DA and GDVII strains. We reached this conclusion because we were able to detect virus antigen and isolate infectious virus from the spinal cords of animals 45 days after inoculation with the same recombinant viruses that produced demyelination.

The above conclusions differ from those of Brahic and colleagues (9, 13). The latter investigators reported recently that recombinant viruses which contained the GDVII leader (L) to 2A coding region within the DA genome failed to persist or cause demyelination in the 10% of mice that survived acute infection (10), a recombinant virus which contained the DA VP1 coding area substituted within the GDVII genome caused demyelination, and a recombinant virus that contained the GDVII VP1 coding area substituted within the DA genome did not cause demyelination in survivors (10). These investigators concluded that the mere attenuation of the neurovirulence of GDVII virus does not allow this virus to persist and, in contrast to our findings (4), they reported that the VP1 protein of DA virus is not only sufficient to allow Theiler's virus to persist and to cause demyelination but also necessary (10). It should be noted that although we believe that both DA and GDVII strains have multiple determinants for demyelination and virus persistence, we have observed that not all DA/GDVII recombinant viruses produced in the laboratory cause demyelination or persist (data not shown). It is clear that recombinant viruses which do not grow at all in the CNS will not cause demyelinating disease and it is probable that some recombinants that grow very poorly and are very attenuated with respect to early CNS infection will clearly not cause the late demyelinating disease. However, it should also be noted that the presence of a vigorous early CNS infection does not ensure the later occurrence of demyelinating disease (30). In addition, there may be some recombinant viruses that have a chimeric protein in which the parental components "fit poorly," putting it at a biological disadvantage and leading to poor virus growth and no late demyelinating disease. It may be that these phenomena led to the results of Brahic and colleagues (10).

Although determinants for demyelination and persistence are present on both DA and GDVII strains, our present studies demonstrate that different TMEV segments can substantially influence the degree of spinal cord demyelination. Infection with the GDVII/DA recombinant viruses appears to prominently affect the degree of spinal cord demyelination, rather than the presence or absence of this white matter disease. Major differences in degree of demyelination were observed when disease was induced by recombinant GD3C-3'/DAFL3 virus, which caused many demyelinated lesions, compared with GD2C-3C/DAFL3 virus, which produced few. Compared with DAFL3 virus the GD 2C-3C/DAFL3 virus has 16 amino acid differences in the following nonstructural proteins: 2C, which is of unknown function; 3AB, which is cleaved to 3B(VPg) and is believed important in viral replication; and 3C, which is the major TMEV proteinase (25). The reason for the decreased amount of late demyelination following inoculation of GD2C-3C/DAFL3 remains unclear. It is possible that changes within the 3C proteinase in the GD2C-3C/DAFL3 virus affect processing of the TMEV polyprotein and thus alter the ability of the virus to persist and demyelinate.

In addition, infection of mice with recombinant GD5'-1B/

DAFL3 virus resulted in a decrease in number of demyelinated lesions compared with DAFL3 or GD1B-2C/DAFL3 viruses. GD5'-1B/DAFL3 virus encodes for 21 amino acid differences in the leader and 1A(VP4) compared with DAFL3 virus. It is important to note that infection with GDNC/DAFL3 virus resulted in a moderate number of demyelinated lesions which were statistically increased in number compared with infection with GD5'-1B/DAFL3 virus, suggesting that the first 280 nucleotides of the GDVII 5' noncoding region or the L or 1A(VP4) coding segment may critically affect the degree of demyelination. Our recent studies have also suggested the importance of the L coding region to demyelination (5a).

Our finding of various demyelinating activities of recombinant TMEV viruses suggest that TO subgroup- or DA strain-specific sequences have an important effect on the degree of late white matter disease. A similar conclusion has been suggested by other studies (11, 13) that have demonstrated the effect of particular DA strain-specific sequences on demyelination. Experiments involving DA neutralization escape mutant viruses have identified DA strain-specific sequences important in the development of the late demyelinating disease. DA virus which contains a change in a single amino acid at either residue 101 or 268 in VP1, part of neutralizing monoclonal antibody sites, produce little if any demyelination (12, 24, 28-30). In these cases, the mutated virus seems to be cleared from the CNS early after infection, and demyelination and virus persistence do not occur. Other variants of DA virus which do not produce demyelination have been reported, although the particular sites for the presumed mutations have not been identified (14, 15); one variant produces little demyelination despite persistence of the virus (14). The molecular basis for the decreased late demyelinating disease following inoculation with the above mutant viruses or for the recombinant viruses we have generated remains unclear. Studies investigating the characterization of the immune response and localization of virus replication may clarify the reasons for this altered biological behavior as well as the pathogenesis of TMEV-induced demyelinating disease.

ACKNOWLEDGMENTS

This research was supported by research grants NS 24180 (M.R.), 1PO1NS21442 (R.P.R.), and 1PO1NS24575 (R.P.R.) from the National Institutes of Health and RG 1878-B1 (M.R.), RG 2174-A-3 (M.R.), and RG 1512-C-4 from the National Multiple Sclerosis Society.

We acknowledge Mabel L. Pierce, Roger L. Thiemann, Teresa Bodwell, Mark Routbort, and Ben Shim for excellent technical assistance. We thank Kathryn Jensen for secretarial assistance and Naraporn Prayoonwiwat for help with the figures.

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