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Genetic Mapping of the Ability of Theiler's Virus To Persist and Demyelinate

ANDRÉS McALLISTER, FRÉDÉRIC TANGY, CHRISTINE AUBERT, AND MICHEL BRAHIC*

Unité Virus Lents, UA 1157 Centre National de la Recherche Scientifique, Institut Pasteur, 75724 Paris Cedex 15, France

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Theiler's virus, a murine picornavirus, is responsible for two different types of disease: strains DA, BeAn, and WW persist for more than a year in the white matter of the central nervous system and cause primary demyelination; strains GDVII and FA, on the other hand, cause an acute encephalitis that kills the host in a matter of days. To map the regions of the viral genome responsible for persistence and demyelination, cDNA clones of the entire genomes of the DA and GDVII strains were constructed and cloned into Bluescript plasmid (A. McAllister, F. Tangy, C. Aubert, and M. Brahic, Microb. Pathogen. 7:381–388, 1989; F. Tangy, A. McAllister, and M. Brahic, J. Virol. 63:1101–1106, 1989). We constructed chimeric viruses obtained by exchanging regions between the cDNA clones. Analysis of the disease phenotypes produced by the chimeric viruses allowed us to map persistence and demyelination to a genome segment coding for the VP1 capsid protein and 27 amino acids of protein 2A.

A number of animal viruses cause persistent infections associated with chronic primary demyelination. These diseases may serve as animal models for the study of multiple sclerosis or other demyelinating diseases. Among them, Theiler's virus infection offers an ideal system to study viral genes responsible for persistence and demyelination. Theiler's virus is a murine picornavirus related to encephalomyocarditis virus and mengovirus, which are members of the *Cardiovirus* genus (16, 17, 19). Strains DA, BeAn, and WW are responsible for a biphasic disease of the central nervous system of susceptible mice (11). The first phase, or early disease, is an acute encephalomyelitis which occurs during the first few days following intracranial inoculation. During this period the virus is found almost exclusively in neurons of the spinal cord. The number of infected cells is small, and most of the animals survive. Soon after, the neurons are cleared of virus and the animals enter a second phase, or late disease, during which the virus is found in the white matter of the spinal cord. This period lasts for over a year and the virus persists in glial cells, mostly oligodendrocytes (2). Late disease is characterized by focal inflammatory lesions where numerous demyelinated axons can be observed (7, 11). These lesions closely resemble those of multiple sclerosis (6, 10). In contrast, strains GDVII and FA are highly virulent. They replicate permissively in neurons, killing their host in a matter of days (24, 26). These two strains are unable to persist or demyelinate (12).

All Theiler's virus strains, however, are closely related. They replicate to high titers in BHK or L cells and have common neutralization epitopes (13). The DA and GDVII genomes have been sequenced. They are 95% homologous at the amino acid level (16–18). Amino acid differences are found throughout the genome but they cluster in proteins L, VP1, and 2A. The 3′ and 5′ noncoding regions are 99.2 and 95.5% homologous, respectively.

We decided to identify viral genes responsible for persistence and demyelination by analyzing the phenotypes of recombinants between the DA and GDVII viruses. In previous articles we reported the construction of infectious cDNA clones for each strain (15, 25). In this paper, we describe the construction of chimeric viruses obtained by exchanging different parts of the genomes. The analysis of the diseases caused by the chimeric viruses allowed us to conclude that the ability to persist and demyelinate maps to a segment of the DA genome coding for capsid protein VP1 and 27 amino acids of the N-terminal extremity of protein 2A.

MATERIALS AND METHODS

cDNA clones and construction of plasmids pTMR2, pTMR3, and pTMR9. The constructions of the cDNA clones of the entire genomes of strains DA and GDVII have been described elsewhere (15, 25). Both constructions were cloned in a modified Bluescript plasmid (pTM) in which the T7 promoter is placed next to the viral cDNA. Plasmids were amplified and purified by centrifugation in CsCl density gradients followed by RNase A and proteinase K digestion, phenol-chloroform extraction, and ethanol precipitation. Purified plasmids were digested with the appropriate restriction enzymes (see Fig. 1). The fragments to be exchanged were purified by agarose gel electrophoresis and Nal-glass bead extraction and ligated to the converse genome in 10-μl reactions containing 50 mM Tris hydrochloride (pH 7.6), 10 mM $MgCl_2$, 5% (wt/wt) polyethylene glycol 8000, 1 mM ATP, 1 mM dithiothreitol, 100 ng of DNA, and 1 U of T4 DNA ligase. The reaction mixtures were incubated for 1 to 6 h at 15°C and used to transform competent DH5α cells as described by Hanahan (8).

In vitro transcription. Plasmids were linearized by digestion with *ClaI* or *XhoI* restriction enzyme (15, 25), treated with proteinase K, extracted with phenol-chloroform, and precipitated with ethanol. RNA transcription was performed with T7 RNA polymerase (Stratagene) in a reaction mixture containing 40 mM Tris hydrochloride (pH 8), 10 mM $MgCl_2$, 2 mM spermidine, 50 mM NaCl, 10 mM dithiothreitol, 0.4 mM each ATP, CTP, UTP, and GTP, 100 U of RNasin, 4 μg of linearized plasmid, and 40 U of T7 RNA polymerase. The reaction was incubated at 37°C for 30 min. The size of the

* Corresponding author.

RNA synthesized (8 kilobases) was analyzed by formamide-formaldehyde agarose gel electrophoresis.

Transfection of cells with RNA. Monolayers of BHK-21 cells grown in Dulbecco modified minimum essential medium (DMEM) supplemented with 10% newborn calf serum were used for transfection experiments. Transfections were carried out as described by Van der Werf et al. (27). RNA was diluted in HBSS (5 g of HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid], 8 g of NaCl, 0.37 g of KCl, 0.125 g of $Na_2HPO_4 \cdot 2H_2O$, 1 g of glucose per liter, pH 7), mixed with an equal volume of DEAE-dextran (1 mg/ml in HBSS), and placed at 0°C for 30 min. Confluent cells were washed three times with DMEM and incubated for 30 min at room temperature with 0.2 ml of the RNA–DEAE-dextran mixture per 35-mm plate. DMEM containing 2.5% fetal calf serum was added, and the plates were incubated for 5 h at 37°C. Cells were then washed three times with DMEM and incubated at 37°C in DMEM without serum for 2 to 3 days. After complete cytopathic effect had occurred, supernatants were harvested and clarified, titers were determined, and supernatants were used to inoculate mice.

Animal studies. SJL/J mice, 3 to 4 weeks old, were purchased from the Institut Pasteur. They were inoculated intracranially with 50 μl of phosphate-buffered saline containing 10^4 PFU of the various viruses. To detect viral RNA and antigens, mice were perfused under anesthesia with 20 ml of phosphate-buffered saline followed by 20 ml of cold paraformaldehyde-glutaraldehyde fixative (3). Dissection of the central nervous system, postfixation, paraffin embedding, and sectioning were carried out as described previously (3). The detection of viral RNA by in situ hybridization was performed as described before (5), using a ^{35}S-labeled RNA probe (10^9 dpm/μg) complementary to 280 nucleotides of the 5' extremity of the viral genome. The detection of Theiler's virus capsid antigens by immunocytochemistry was performed on central nervous system paraffin sections as described previously (3), using an immune serum which binds strongly to capsid proteins VP1 and VP2 and weakly to capsid protein VP3. For myelin studies, animals were perfused with a fixative containing 4% paraformaldehyde and 1% glutaraldehyde. The myelin staining was performed with toluidine blue on sections of Epon-resin-embedded spinal cord.

RESULTS

Sequence comparison of DA and GDVII genomes. The sequences of the genomes of strains DA, BeAn, and GDVII have been published (16–19). Figure 1 shows the positions of those amino acids which are identical in DA and BeAn but different in GDVII. It can be seen that differences are more frequently found in proteins L, P1, and 2A than in the rest of the coding genome. Since both DA and BeAn are able to persist and demyelinate, these amino acids are more likely to play a role in persistence and demyelination. Therefore, in a first step, we decided to study the phenotypes of chimeric viruses in which these regions had been exchanged.

Persistence and demyelination maps within the *Aat*II/*Aat*II fragment. Figure 1 shows the map of the chimeric plasmids used in this study. Plasmids pTMR2 and pTMR3 consist of an exchange of an *Aat*II-*Aat*II restriction fragment between parental plasmids pTMDA and pTMGDVII. This fragment codes for 30 amino acids of the C-terminal extremity of the L protein, the entire capsid, and 27 amino acids of the N-terminal extremity of protein 2A. Full-length, positive-strand cRNAs were synthesized for both plasmids, and

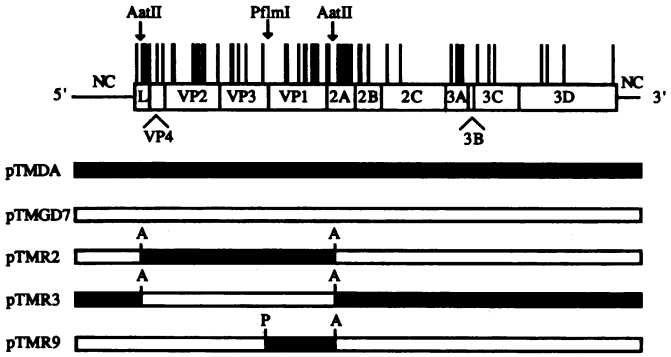

FIG. 1. Schematic representation of amino acid differences among three strains of Theiler's virus and genome structure of recombinants between DA (■) and GDVII (□) virus sequences. The genomic organization of Theiler's virus RNA is indicated in the standard nomenclature. The positions of those amino acids which are identical in DA and BeAn but different in GDVII are shown by vertical bars. The number of differences within the *Aat*II-*Aat*II fragment is 31 for 902 amino acids, whereas it is only 22 for 1,354 amino acids in the rest of the coding region. Differences in the noncoding (NC) regions are not shown. A and P represent cleavage sites of the restriction enzymes *Aat*II and *Pfl*mI, respectively.

BHK cells were transfected with 20 μg of each RNA. Both were infectious and gave cytopathic effect after 2 to 3 days of incubation. The titers obtained following transfection were 5×10^6 and 6.6×10^5 PFU/ml, respectively.

SJL/J mice were inoculated intracerebrally with 10^4 PFU of parental or chimeric viruses. The phenotypes of chimeric viruses are summarized in Table 1, and some features of the histopathology are shown in Fig. 2. In all cases, viral replication and histopathology were evaluated by a combination of in situ hybridization, immunocytochemistry, and myelin staining on Epon semithin sections as described previously (15, 25). A neurovirulent, GDVII-like phenotype was defined by high mortality during the first 7 days following inoculation and by acute grey matter encephalitis with large amounts of viral RNA and antigens present in neurons of brain and spinal cord. On the other hand, a persistent and demyelinating, DA-like phenotype was defined by the following criteria: (i) the absence of mortality in SJL/J mice; (ii) the presence of viral RNA and antigens in glial cells of the white matter 45 days postinoculation; (iii) the association of these infected cells with inflammatory infiltrates; and (iv) the presence, in semithin sections, of numerous demyelinated axons.

Table 1 and Fig. 2 show that the phenotype of chimeric virus R2 was indistinguishable from that of the parental DA virus. In particular, intense inflammation of the white matter surrounding infected glial cells and prominent demyelination

TABLE 1. Phenotype of parental and chimeric viruses

Virus	% Mortality[a]	Persistent infection of white matter	Demyelination
DA	0	+	+
GDVII	100	−	−
R2	0	+	+
R3	90	−	−
R9	0	+	+

[a] Twenty-four mice were inoculated with each virus. Mortality due to acute grey matter encephalomyelitis occurred within 10 days postinoculation. No mice died after that time.

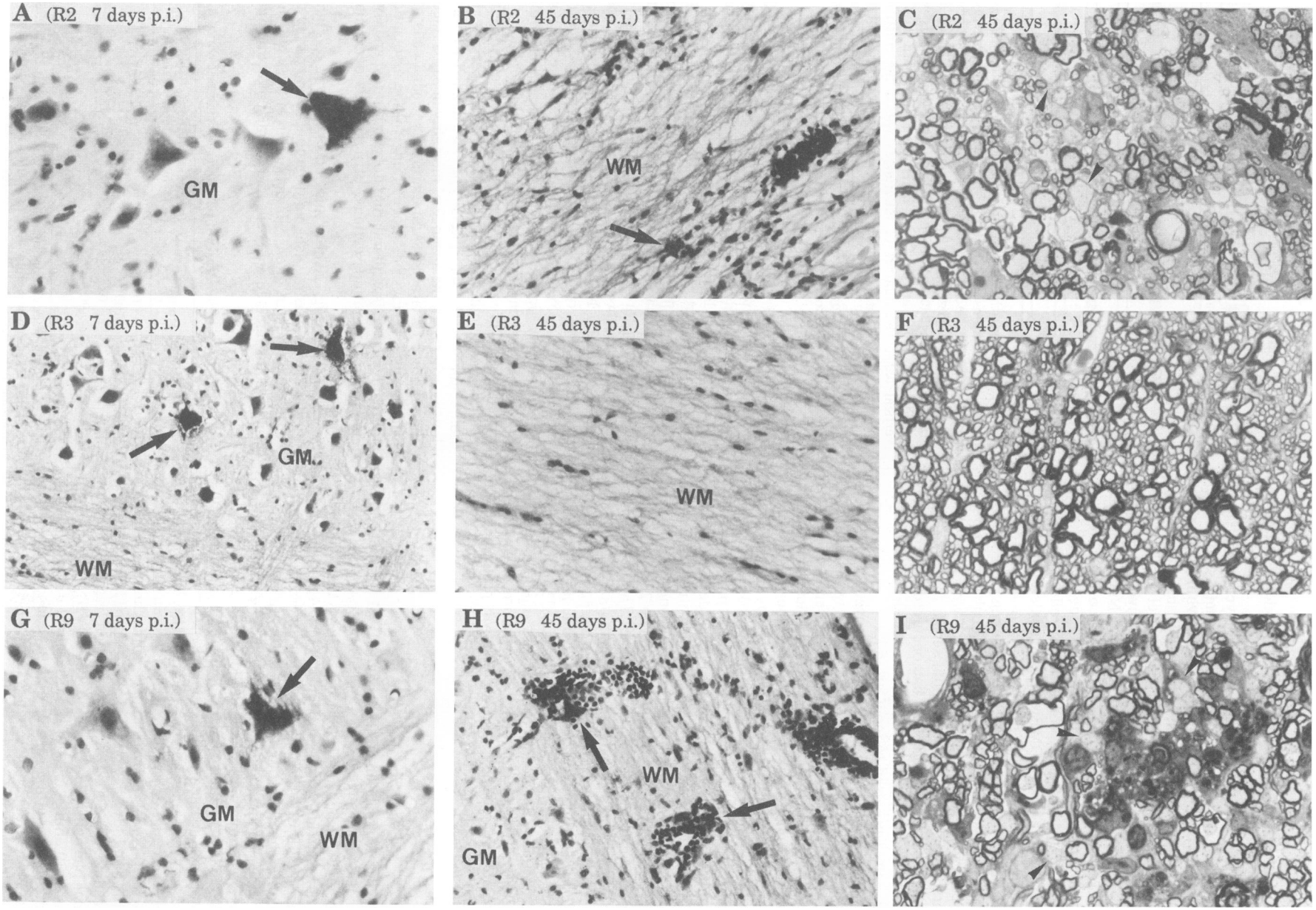
A (R2 7 days p.i.)
GM
B (R2 45 days p.i.)
WM
C (R2 45 days p.i.)
D (R3 7 days p.i.)
GM
WM
E (R3 45 days p.i.)
WM
F (R3 45 days p.i.)
G (R9 7 days p.i.)
GM
WM
H (R9 45 days p.i.)
WM
GM
I (R9 45 days p.i.)

FIG. 2. Histopathological findings at different times postinfection (p.i.) with R2, R3, and R9 viruses: detection of Theiler's virus capsid antigens by immunocytochemistry on longitudinal sections of paraffin-embedded spinal cord, and myelin staining with toluidine blue on transversal sections of Epon-embedded spinal cord. SJL/J mice were inoculated intracerebrally with 10^4 PFU of virus R2 (A, B, and C), R3 (D, E, and F), or R9 (G, H, and I). Mice were sacrificed 7 (A, D, and G) or 45 (B, C, E, F, H, and I) days postinoculation. Seven days postinoculation, animals infected with R2 and R9 showed a small number of infected neurons (A and G), whereas animals infected with R3 (D) showed extensive areas of a highly lytic infection in grey matter (GM). Arrows point to cells containing large amounts of viral antigens. Mice sacrificed 45 days postinoculation with viruses R2 and R9 (B and H) showed a number of cells in the white matter (WM) containing viral antigens (arrows). These cells are surrounded by inflammatory infiltrates. At this time, myelin staining showed extensive demyelination (C and I). Arrowheads point to some of the numerous demyelinated axons. On the contrary, at the same time point (45 days postinoculation), no antigen or inflammation was found in tissue of mice infected with R3 virus (E), and myelin staining showed no abnormality in any of the mice studied (F). Magnification: ×1,823 (panels A, C, F, G, and I), ×737 (panels B, E, and H), and ×460 (panel D).

were observed 45 days postinoculation. There was no mortality within this series of mice. On the other hand, chimeric virus R3 behaved almost like a parental GDVII virus. The only difference was a reduction of mortality rate from 100 to 90%. Seven days postinfection, both grey and white matter were infected as detected by in situ hybridization and immunocytochemistry and by the presence of large inflammatory infiltrates (Fig. 3). The survivors (six mice) were sacrificed 45 days postinoculation. Despite the fact that they showed residual flacid paralysis of the hind legs, viral RNA, viral antigens, inflammation, or demyelination were not found in the central nervous system (Fig. 2). Therefore, the *Aat*II-*Aat*II fragment of the GDVII genome contains most, but not all, of the information necessary for the neurovirulence of this strain. Furthermore, virus R3 was not able to persist or demyelinate even though it was able to infect the white matter (Fig. 3). This finding demonstrates that both DA and GDVII capsids are able to infect the same cell types and that mere attenuation does not determine the ability to persist and demyelinate.

These findings allowed us to conclude that all information required for persistence and demyelination of the DA strain resides within the *Aat*II-*Aat*II fragment of the genome.

It should be noted that chimera R2 is the equivalent of the GDVII/BeAn chi-3 chimera described by Calenoff et al. (4). These authors reported that chi-3 showed attenuated virulence, in agreement with our findings with chimera R2. However, since the authors did not address the question of persistence and demyelination, a comparison of R2 and chi-3 chimeras is not possible in this respect.

Persistence and demyelination maps within the *Pfl*mI-*Aat*II fragment. Within the L-P1-2A region, capsid protein VP1 is a prime candidate for the determinant of persistence and demyelination. First, VP1 is known to play a major role in antigenicity and viral tropism of picornaviruses (23). Second, in the case of Theiler's virus, two neutralizing antibody escape mutants with altered pathogenicity have been described (22, 28, 29). In both cases, the putative mutation was mapped to capsid protein VP1.

Therefore, to test the role of VP1 in persistence and demyelination, we constructed chimeric plasmid pTMR9 (Fig. 1). Chimera R9 consists of an exchange of a *Pfl*mI-*Aat*II fragment which codes for 6 amino acids of VP3, the entire VP1, and 27 amino acids of protein 2A. Of the six VP3 amino acids exchanged, only one is different between DA and GDVII. It is likely that this difference will not be a determinant of persistence and demyelination since it does not exist between GDVII and BeAn. The *Aat*II site located at the 5′ end of the 2A gene was the same site used previously for the construction of pTMR2 and pTMR3.

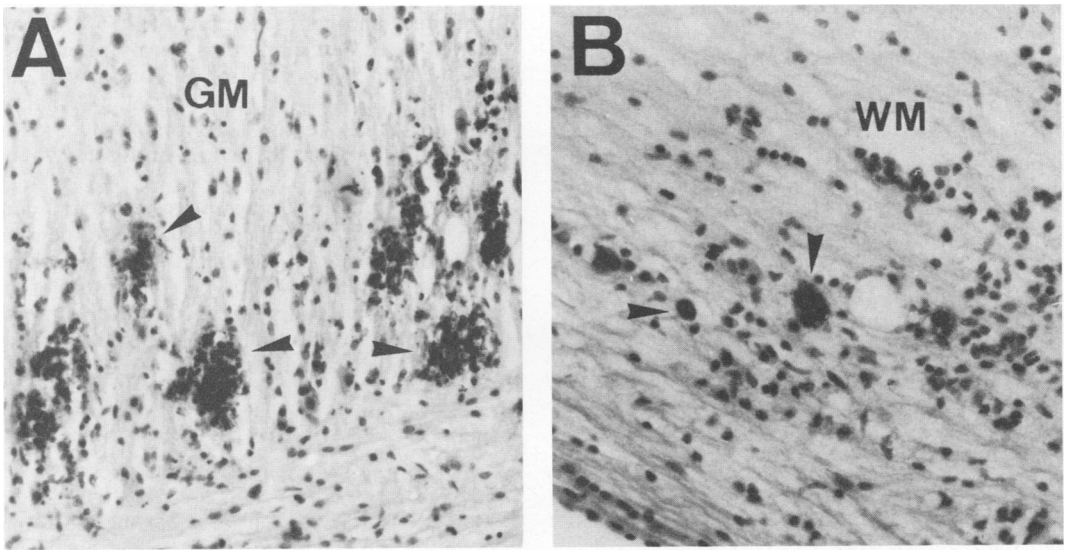

FIG. 3. Histopathological findings 7 days postinfection with R3 virus. SJL/J mice were inoculated intracerebrally with 10^4 PFU of virus R3 and sacrificed 7 days postinoculation. Theiler's virus capsid antigens were detected by immunocytochemistry on longitudinal sections of paraffin-embedded spinal cord. (A) Example of grey matter (GM) area with lytic infection of neurons typical of the neurovirulent (GDVII) strain of Theiler's virus. Arrows point to cells expressing large amounts of antigen. (B) Example of white matter (WM) area with cells containing viral antigens and surrounded by inflammatory infiltrates. Magnification, ×2,560.

Full-length, positive-strand cRNA was synthesized from pTMR9 plasmid, and 20 μg was used to transfect BHK cells. A complete cytopathic effect was observed 48 h posttransfection, and the titer of the virus obtained was 2×10^7 PFU/ml. SJL/J mice were inoculated intracerebrally with 10^4 PFU of R9. Table 1 shows that the phenotype of chimeric virus R9 was identical to those of R2 and parental DA viruses. The virus did not kill mice and was able to persist and to induce intense inflammation and demyelination in the white matter (Fig. 2). These results demonstrated that the VP1 capsid protein and/or 27 amino acids at the N-terminal extremity of protein 2A of the DA genome are sufficient to allow viral persistence and demyelination. They also show that the same region of the DA genome is sufficient to attenuate fully the neurovirulence of GDVII virus.

DISCUSSION

Theiler's virus offers an ideal system to study viral genes important in pathogenesis. Although strains DA and GDVII share a high degree of sequence homology, they cause two entirely different neuropathologies. Using genomic cDNA clones of these two strains, we produced a series of recombinant chimeras that allowed us to map viral genes responsible for the different phenotypes. Two aspects can be studied with this approach: first, persistence and demyelination of the DA strain; and second, neurovirulence of the GDVII strain. The aim of the present work was to identify viral genes responsible for persistence in the central nervous system and for the accompanying demyelination observed during chronic disease.

The first part of our results, the study of chimeras R2 and R3, demonstrated that the L-P1-2A coding sequence of the DA genome contains genes that allow the virus to persist and demyelinate. If other genes outside these boundaries play a role, they must be present in both genomes and they cannot entail persistence and demyelination by themselves. The second part of our results, the study of chimera R9, showed that introducing the VP1 and the N-terminal extremity of protein 2A of DA into the GDVII genome produced a chimera with a DA-like phenotype. It is likely that the DA phenotype is due to capsid protein VP1 and not to protein 2A, since within the 27 amino acids of protein 2A concerned by this construction, only two amino acid changes are common to DA and BeAn viruses (Ser-926 ⇒ Ala and Leu-940 ⇒ Phe). If these two amino acids are responsible for the change of phenotype, it is impossible to explain their role at present since the function of protein 2A is still unknown (21).

In contrast, different mechanisms can be proposed to explain the role of VP1.

(i) Because VP1 contains part of the putative receptor binding site in all picornaviruses studied (1, 9, 14, 23), its replacement may affect cell tropism. However, tropism alone does not explain the difference of neuropathology caused by the two strains since R3 chimera is able to infect both neurons and glial cells (Fig. 3). This observation is compatible with the predicted structure of Theiler's virus capsid. According to this model, the putative viral receptor binding sites of GDVII and BeAn viruses are markedly conserved (20).

(ii) Antigenic differences in VP1 could bring about changes in the immune response which could determine persistence and demyelination. A comparison of the predicted structure of the capsid proteins of GDVII and DA viruses shows that most of the differences are clustered in the loops and corners connecting β-strands. In the case of poliovirus, rhinovirus, and mengo virus, these regions have been identified as major neutralizing immunogenic epitopes. For VP1, the "third corner" between β-strands D and E differs markedly between GDVII and DA strains. These differences could entail changes in the immune response which would be responsible for the change in phenotype. Other clusters of differences are found in the putative major neutralizing immunogenic epitopes of VP2 and VP3. Whether these differences are also able to produce persistence and demyelination independently of those of VP1 remains to be established.

(iii) A hallmark of Theiler's virus persistent infection is restricted viral replication in glial cells. VP1 could also play its role in persistence by restricting, through an unknown mechanism, virus expression in glia.

Regardless of the mechanism by which VP1 causes persistence and demyelination, this property is specific to DA virus. Mice which survive the infection by R3 chimera, or by GDVII (12), are not persistently infected and do not demyelinate. Therefore, the phenotype is not acquired by simply attenuating GDVII virus.

The study of the neurovirulence of GDVII may require the use of fine measurements of attenuation, such as 50% lethal dose. Since our main interest was to map genes determining persistence and demyelination, we did not carry out this type of analysis. However, some conclusions regarding the neurovirulence of GDVII can be drawn from our study. We observed that the replacement of the *Aat*II-*Aat*II fragment of GDVII by that of DA fully attenuated chimera R2. Conversely, by introducing the corresponding region of GDVII into DA, chimera R3 acquired most, but not all, of the neurovirulence of GDVII. This result shows that most, but not all, of the neurovirulence determinants of GDVII map within the *Aat*II-*Aat*II fragment. Furthermore, chimera R9 is still attenuated although it contains only the *Pfl*mI-*Aat*II fragment of the DA genome. This suggests that the VP1/2A region of GDVII contains a determinant of neurovirulence, although it is probably not the only one within the *Aat*II-*Aat*II fragment. Neurovirulence is likely to be caused by several determinants scattered along the genome. In fact, we produced another recombinant (pTMR4) which demonstrated that a determinant of neurovirulence is located in the region coding for part of the L protein, VP4, and part of VP2 (unpublished data). Calenoff et al. (4) mapped neurovirulence primarily to the L-P1 region. Our data suggest that within this region there are at least two neurovirulence determinants.

The present work maps persistence and demyelination to the *Pfl*mI-*Aat*II fragment of the DA genome. It will be important to identify within this region shorter fragments responsible for the phenotype. The nature of the corresponding peptides and, in particular, their locations on the three-dimensional structure of the capsid will yield important information on the mechanism of persistence and demyelination.

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