JOURNAL OF VIROLOGY, May 1994, p. 3364–3368
0022-538X/94/$04.00+0

Vol. 68, No. 5

NOTES

A Single Amino Acid Change Determines Persistence of a Chimeric Theiler's Virus

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Received 29 October 1993/Accepted 10 January 1994

The DA strain of Theiler's virus persists in the central nervous system of mice and causes chronic inflammation and demyelination. On the other hand, the GDVII strain causes an acute encephalitis and does not persist in surviving animals. Series of recombinants between infectious cDNA clones of the genomes of DA and GDVII viruses have been constructed. The analysis of the phenotypes of the recombinant viruses has shown that determinants of persistence and demyelination are present in the capsid proteins of DA virus. Chimeric viruses constructed by the different research groups gave consistent results, with one exception. Chimeras GD1B-2A/DAFL3 and GD1B-2C/DAFL3, which contain part of capsid protein VP2, capsid proteins VP3 and VP1, and different portions of P2 of GDVII in a DA background, were able to persist and cause demyelination. Chimera R4, whose genetic map is identical to that of GD1B-2A/DAFL3, was not. After exchanging the viral chimeras between laboratories and verifying each other's observations, new chimeras were generated in order to explain this difference. Here we report that the discrepancy can be attributed to a single amino acid difference in the sequence of the capsid protein VP2 of the two parental DA strains. DAFL3 (University of Chicago) and the chimeras derived from it, GD1B-2A/DAFL3 and GD1B-2C/DAFL3, contain a Lys at position 141, while TMDA (Institut Pasteur) and R4, the chimera derived from it, contain an Asn in that position. This amino acid is located at the tip of the EF loop, on the rim of the depression spanning the twofold axis of the capsid. These results show that a single amino acid change can confer the ability to persist and demyelinate to a chimeric Theiler's virus, and they pinpoint a region of the viral capsid that is important for this phenotype.

Viral recombinants between related viruses are now widely used in order to determine and study the function of viral genes. Several years ago, we decided to take this approach in order to study the pathology produced by Theiler's virus infection of the central nervous systems (CNS) of certain strains of mice. Theiler's virus is a picornavirus related to encephalomyocarditis virus and to mengo virus, which are members of the *Cardiovirus* genus (17, 18, 20). The DA strain of Theiler's virus is responsible for a biphasic disease of the CNS 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 CNS. 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. During this period the virus persists in oligodendrocytes (1) and in macrophages (5). Late disease is characterized by focal inflammatory lesions around infected cells where numerous demyelinated axons can be observed (7, 11). These lesions resemble closely those of multiple sclerosis (6, 10). In contrast, strain GDVII is highly virulent. It replicates permissively in neurons and kills its host from severe encephalitis in a matter of days (24, 27). In the few survivors, GDVII is unable to persist or demyelinate (12). Both strains, however, are closely related. They replicate to high titers in BHK or L cells, share common neutralization epitopes, and have overall sequence homologies of 90% at the nucleotide level and 95% at the amino acid level (17–20).

In order to map viral genes responsible for persistence and demyelination, a series of recombinants had been constructed by exchanging restriction fragments between cDNA clones containing the entire sequences of strains GDVII and DA (15, 22, 26). The disease phenotypes produced by the recombinants indicated that the viral capsid, in particular protein VP1, of strain DA and/or the 27 N-terminal amino acids of protein 2A played a central role in determining viral persistence and demyelination (16, 25). However, the study of another series of recombinants did not come to this conclusion, since two of the chimeras were able to persist and produce demyelination despite the fact that most of their capsid proteins, including

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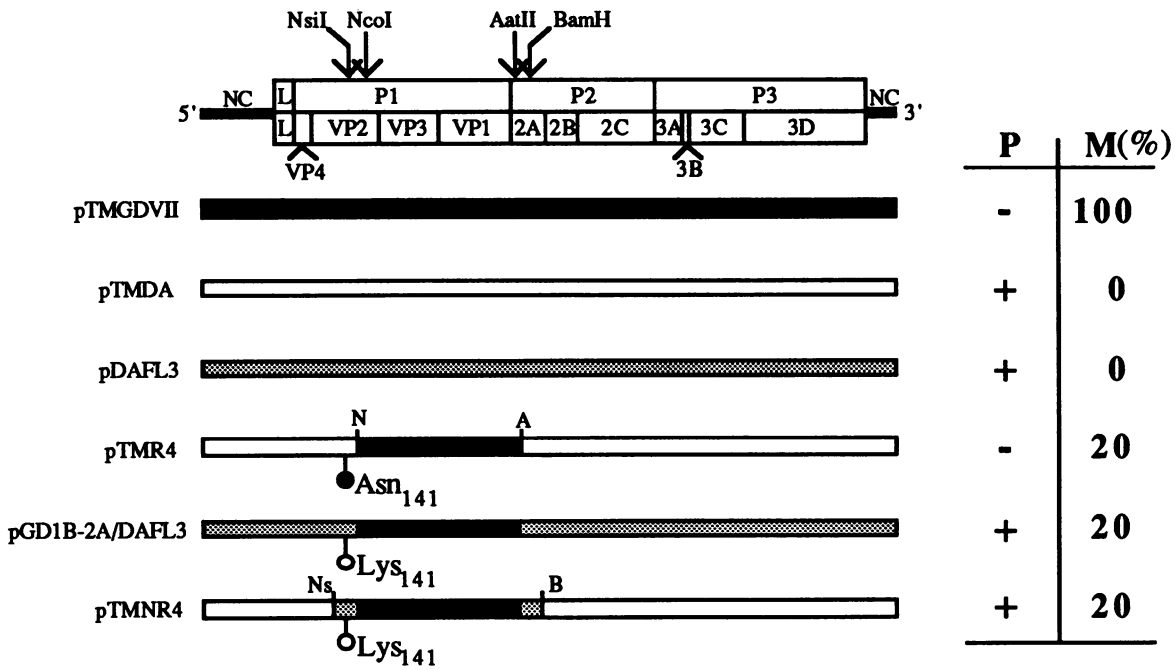

FIG. 1. Schematic representation of the genomic organization of Theiler's virus RNA and position of the restriction sites used to construct the recombinant cDNAs. Genetic map of the different recombinants constructed from pTMGDVII (■), pTMDA (□), and pDAFL3 (▩). N, A, Ns, and B, cleavage sites of the restriction enzymes *Nco*I, *Aat*II, *Nsi*I, and *Bam*HI, respectively. The figure also shows a summary of the phenotypes of viruses DA, GDVII, R4, GD1B2A/DAFL3, and NR4. P, persistence of infection; M, mortality.

VP1, came from virus GDVII (Fig. 1) (8). After constructs were exchanged between the two laboratories, the conflicting results were reproduced by the two groups, ruling out that the discrepancy was due to a trivial cause, such as a difference in the amount of virus used for inoculation or in the sensitivity of viral antigen detection by immunocytochemistry. Therefore, it became evident that the difference had to be explained at the genomic level.

To test whether the persisting phenotype was due to the GDVII portion of the capsid used in chimera GD1B2A/DAFL3, a new chimeric virus, pTMNR4, was constructed (Fig. 1). Briefly, the *Nsi*I-*Bam*HI restriction fragment of pGD1B2A/DAFL3, shown in Fig. 1, which codes for part of VP2, VP3, and VP1 and part of protein 2A, was purified by gel electrophoresis and exchanged for its counterpart of plasmid pT MDA. In this way, a full-length, infectious cDNA in which the T7 promoter is placed next to the viral cDNA was generated (26). As described previously, full-length RNA was synthesized with T7 RNA polymerase, with the linearized recombinant plasmid as a template (15). Monolayers of BHK-21 cells were transfected by electroporation with in vitro-transcribed RNA (2). After complete cytopathic effect had occurred, supernatants were harvested and viral titers were determined by a standard plaque assay with BHK-21 cells. Viruses R4 and NR4 grew to similar titers and produced large plaques that were indistinguishable from one another.

Two groups of five 3-week-old SJL/J mice were inoculated intracranially with 50 μl of phosphate-buffered saline containing 10^4 PFU of R4 or NR4 virus. Mice were observed daily to record mortality. CNS histology and detection of viral antigens by immunocytochemistry were performed as described before (1). A persistent-demyelinating phenotype was defined as the presence of viral antigen and inflammation in the white matter of SJL/J mice 45 days postinoculation. Micrographs illustrating these phenotypes have been published previously (15, 16, 25, 26).

One mouse of each group died during the second week postinfection. As shown in Fig. 1, the mortality produced by the two viruses was the same and it was similar to the one published previously for virus R4 (25). Surviving animals were sacrificed 45 days postinoculation. The histological analysis of the CNS showed that all surviving mice inoculated with virus R4 had completely cleared the virus, since no viral antigen could be detected in serial sections of the brain tissue and spinal cords of these animals. No inflammation was observed in three of them. In the CNS of the fourth animal, a single, small focus of inflammation was seen but no viral antigen could be detected. This result is in agreement with our previous observations (25). On the contrary, the four surviving mice inoculated with virus NR4 showed foci of intense inflammation around areas where large amounts of viral antigen were detected by immunoperoxidase. In fact, the histological phenotype produced by this virus was indistinguishable from that of the parental DA virus.

To confirm this finding, we compared the levels of viral RNA present in the CNS of infected mice 45 days after inoculation with a quantitative filter hybridization assay (3). For this purpose, four groups of SJL/J mice were inoculated with viruses DA, R4, NR4, and R5 (Fig. 2). DA (the positive control) is the parental strain, and R5 (the negative control) is a virus that is cleared from the CNS very soon after infection (25). Surviving animals were sacrificed 45 days postinoculation, and total spinal cord RNA was extracted by the method of Chomczynski and Sacchi (4). A quantitative dot blot hybridization analysis was carried out as described before (3). Briefly, a series of fivefold dilutions of total RNA were dotted on a Hybond C^+ membrane (Amersham), starting with 10 μg of total RNA per dot, and hybridized with a probe containing

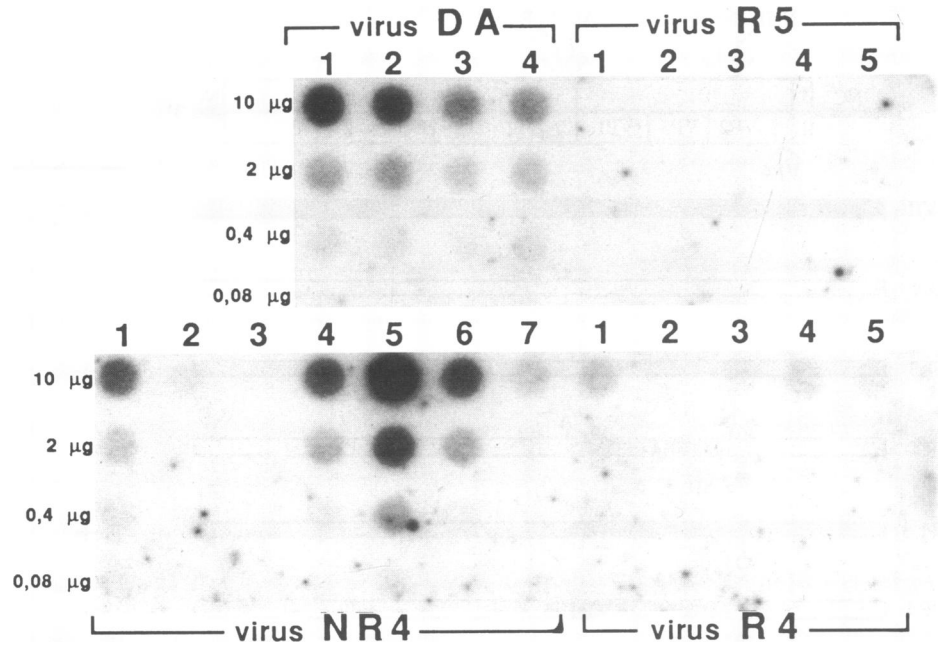

FIG. 2. Dot blot hybridization analysis of total RNA extracted from the spinal cord of infected mice, 45 days postinoculation. Mice were inoculated with 10^4 PFU of virus DA, R5, R4, or NR4. Series of fivefold dilutions of total RNA were dotted on a Hybond C^+ membrane (Amersham), starting with 10 µg of total RNA per dot and hybridized with a ^{32}P-labeled probe containing 2,011 nt of viral sequence. The integrity of the RNA was demonstrated by hybridizing the same RNAs with a β-actin probe (data not shown).

2,011 nucleotides (nt) of viral sequences radiolabeled with [α-^{32}P]dCTP by random priming of cDNA fragments. The result of this experiment confirmed the one obtained by immunocytochemistry. Viruses R4 and R5 were virtually cleared from the CNS of all mice, whereas virus NR4 was able to persist in the CNS of four of seven infected mice at a level similar to that of virus DA (Fig. 2). This heterogeneity was not due to differences in the integrity of the RNA as demonstrated by hybridization with a β-actin probe (data not shown).

In order to identify the mutation(s) responsible for the difference in phenotype produced by viruses R4 and NR4, the relevant regions of the two infectious cDNAs (nt 1856 to nt 3932) were sequenced. Only one nucleotide difference was found between the two fragments: nt 1929 is a G in virus NR4 and a T in virus R4. This difference changes amino acid 141 of VP2: a Lys in NR4 becomes an Asn in R4. The Asn was found in pTMDA and in chimeras derived from it. On the other hand, the Lys is present in pDAFL3 and in chimeras derived from it. These results were obtained independently in both laboratories. Since the genomes of viruses R4 and NR4 were constructed with the same background (pTMDA) and differ only in the origin of the *Nsi*I-*Bam*HI restriction fragment (Fig. 1), this single amino acid change can bring about persistence of chimera NR4.

To test whether viral particles present in the CNS of persistently infected mice contained the Lys at position 141 of VP2, cDNA was synthesized from the RNA extracted from the spinal cord of one mouse infected with NR4. The synthesis was carried out by random priming with synthetic hexamers and incubation with avian reverse transcriptase (2). The cDNA was then amplified by PCR with two oligonucleotides that generated a band of 942 bp (nt 1622 to nt 2564). Direct sequencing of the PCR products showed that the Lys was indeed present. Conversely, Asn was found at this position in viral RNA extracted from BHK-21 cells infected with a chimera whose VP2 gene came from pTMDA.

The structure of the DA strain has been determined by X-ray crystallography (9). Residue 141 of VP2 is located in the EF loop, a large loop connecting the E and F beta strands of the beta-barrel core of VP2. This large loop contains two distinct, smaller loop structures. The first of these smaller loops, residues 136 to 150, forms a compact twisted beta hairpin. Residue 141 is the first of the four residues which make up the beta turn at the tip of the hairpin. The electron density for residues 142 and 143 is weak and suggests that the backbone takes on more than one conformation at the tip. In the molecular model of DA, the side chain at position 141 was built as a Lys, consistent with the published sequence (17). However, there is no electron density for atoms beyond the gamma carbon, and the shape of the side chain density is consistent with an Asn. A Lys in this position would be very likely to interact with Asp-144, on the far side of the beta turn, or with the neighboring carbonyl oxygen of Pro-147. Either interaction might stabilize the side chain conformation, producing interpretable density for the distal atoms of the side chain. This suggests that the sequence in the virus crystallized includes an Asn, or possibly a mixture of Asn and Lys, at position 141.

The beta hairpin containing VP2 141 is one of four small loop structures that form the upper surface at the tips of the star-shape plateaus centered on the fivefold axes of the virus (Fig. 3 and 4). The others are the second part of the EF loop of VP2 (residues 164 to 181) and two regions of the CD loop of VP1 (residues 78 to 85 and 94 to 110). The first VP2 EF loop interacts with the second VP2 EF loop and with the GH loop of VP1, which lies underneath. The second VP2 EF loop also interacts with the second VP1 CD loop. The first VP1 CD loop is freestanding (9). These four small loops are the most highly exposed features on the viral surface, readily accessible for interaction with antibodies or cell receptors. Any alteration of these loops could change the way they interact with each other

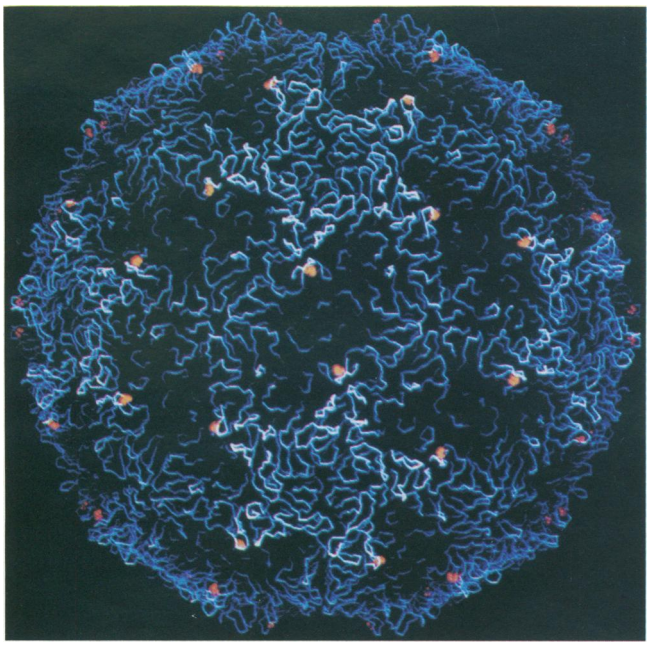

FIG. 3. External surface of Theiler's virus. Except for the side chain atoms of VP2 141, shown as orange spheres, only the alpha carbons are shown. The alpha carbons are colored in a continuous spectrum from dark blue (low radius) to white (high radius) according to their distance from the center of the virus (radial depth cuing).

and modify the surface that the capsid presents to the external environment.

Up to now determinants of viral persistence were known to be located in the viral capsid (16, 25). Further defining the determinants within the capsid had been rather difficult for several reasons. First, many constructs aimed at evaluating the role of the loops that decorate the viral surface are not infectious (unpublished data). Second, in certain cases, assembly defects interfere with the replication of chimeras with hybrid capsid proteins (21). In the present work, we produced two viruses, R4 and NR4, which are dramatically different in their ability to persist and which differ by a single amino acid at position 141 of VP2.

Interestingly, two monoclonal escape mutants of DA virus that are attenuated in their ability to persist and demyelinate carry mutations that have been located in the same area as VP2 141. The first carries a Thr→Ile mutation at position 101 of VP1, a position that is highly exposed at the tip of the CD loop (29, 30). The second bears a Val→Phe change in residue 268 of VP1 (23). This residue is located in the disordered C terminus of VP1 which emerges from the interior of the capsid at the bottom of the twofold axis depression (Fig. 4).

One can think of several roles for amino acid 141 in viral persistence. The extreme ends of the elongated depression spanning the twofold axis are located between the arms of the star-shape plateau. This region corresponds to the feature referred to as the pit in mengo virus (14). It has been suggested that this feature is the receptor binding site for mengo virus and Theiler's virus (9, 13, 14). Residues 101 of VP1 and 141 of VP2 are located on top of the plateau at the rim of the depression. Though they are not likely to affect the interaction of a receptor with the bottom of the depression, they could affect interactions of receptors with the edge of the plateau, if the depression is in fact the receptor binding site. Interactions of this sort may be involved in determining the tissue tropism of the virus, in particular its ability to enter and infect the white matter of the spinal cord. The clustering of important residues in the three-dimensional structure strongly suggests that the role of VP2 141 in persistence is a result of a receptor-mediated effect. Because of the polyclonal nature of the B-cell response, it seems unlikely that a single amino acid substitution in this highly exposed region would abrogate enough of the total B-cell response to prevent clearance. The T-cell response can be directed at any part of any viral protein, and clustering of important epitopes in the three-dimensional structure of the capsid would be fortuitous. Interestingly, in the case of the VP1 101 mutation, studies in nude mice also indicate that reduced

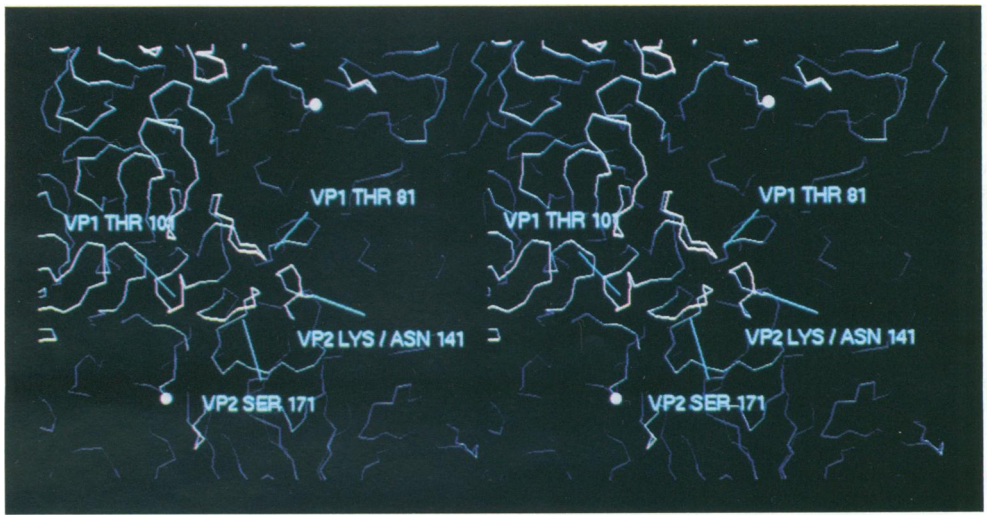

FIG. 4. Close-up stereo view of the EF loop of VP2 and the CD loop of VP1. Both loops contain two smaller loops described in the text. The four smaller loops form the tips of the star-shape plateaus at the fivefold axes of the virus (Fig. 3). This alpha carbon trace of the capsid proteins has the same radial depth cuing as Fig. 3, with atoms at the lowest radius disappearing into the black background. Residues at or near the tips of each small loop are labeled. The last ordered residue (Val-256) of each of the three visible C termini of VP1, emerging from the bottom of the depression, is marked with small white spheres.

ability to persist is not wholly due to a change in B- or T-cell epitopes (31). Obviously, a combination of any of the three mechanisms is possible.

To our surprise, the Asn→Lys mutation responsible for the persistence of virus NR4 came from virus DA and not from virus GDVII. Since VP2 141 is an Asn in pTMDA, Asn at this position is compatible with persistence. This could indicate that the capsid of virus DA bears other determinants of persistence which, by themselves, are sufficient to give this virus its persistent phenotype. This interpretation is consistent with preliminary data showing that a derivative of pTMDA has a lower degree of persistence than pDAFL3. It could also explain why virus NR4, whose capsid is derived mostly from virus GDVII, did not persist in all infected mice (Fig. 2). As has been pointed out by others (21, 28), our results illustrate the difficulty encountered in interpreting data obtained with picornavirus chimeras, a difficulty which is increased when parental stocks of virus in different laboratories differ slightly. In any event, determining one precise amino acid which is able to confer persistence will provide a valuable tool to study the mechanisms of viral persistence. Furthermore, this amino acid helped define a region of the capsid which appears to be critical for this phenotype.

We thank J. F. Bureau for helpful discussions.

This work was supported by grants from Association pour la Recherche sur la Sclérose en Plaques, Centre National de la Recherche Scientifique, Institut Pasteur Foundation, and the National Multiple Sclerosis Society. T.M. was the recipient of an EMBO long-term fellowship and is now a research associate of the National Fund for Scientific Research (Belgium).

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