ALTERATION OF AMINO ACID 101 WITHIN CAPSID PROTEIN VP-1 CHANGES THE PATHOGENICITY OF THEILER'S MURINE ENCEPHALOMYELITIS VIRUS

By ANDREAS ZURBRIGGEN,* JAMES M. HOGLE,‡ and ROBERT S. FUJINAMI*

*From the *Department of Pathology, University of California, San Diego, La Jolla, California 92093; and the ‡Department of Molecular Biology, Research Institute of Scripps Clinic, La Jolla, California 92037*

Theiler's murine encephalomyelitis virus (TMEV)[1] is a member of the picornavirus family. The icosahedral capsid consists of 60 capsomers, each containing a single copy of the four capsid proteins: VP-1, VP-2, VP-3, and VP-4. The genome, ~8,100 nucleotides long, is a single-stranded RNA of positive polarity with more than a 1,000-base-long 5′ noncoding region and a poly(A) tail at the 3′ end (1). Nucleotide sequence analysis has revealed that TMEV is closely related to the cardioviruses, i.e., encephalomyocarditis (EMC) and Mengo viruses (2–4).

TMEV is a naturally occurring murine virus that leads to an asymptomatic enteric infection in most young adult mice. In rare instances, a spontaneous central nervous system (CNS) disease has been observed (5). Since its original description a number of different viral strains have been isolated. The strains can be divided into two subgroups based on their pathologic and biologic characteristics. The GD VII/FA subgroup is highly virulent and produces an acute polio encephalitis that kills the host. In contrast, members of the TO (Theiler's original) subgroup, which include the DA strain (6), produce a biphasic disease in susceptible mice (7). The acute disease is similar to human poliomyelitis and is characterized by virus replication, inflammation, necrosis of neurons, and glial cell proliferation (8). Survivors of the acute phase go on to develop the secondary chronic disease that is marked by spasticity of limbs (7). The relative severity of the acute versus chronic disease is dependent on the genetic background of the mice (9). In SJL/J mice, most of the infected animals survive the acute disease without exhibiting severe clinical symptoms (7).

The pathological features of the chronic disease include extensive meningeal and perivascular infiltrates with concomitant demyelination (10). The cellular infiltrates

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Address correspondence to Dr. Robert S. Fujinami, Dept. of Pathology, M-102, University of California, San Diego, School of Medicine, La Jolla, CA 92093-0612.

[1] *Abbreviations used in this paper:* CNS, central nervous system; EMC, encephalomyocarditis; PFU, plaque-forming units; TMEV, Theiler's murine encephalomyelitis virus; TO, Theiler's origin.

in the CNS are comprised of macrophages, lymphocytes, monocytes, and plasma cells (8). During the chronic stage of the disease most pathologic processes are limited to the CNS (11). The demyelination parallels incomplete CNS-type remyelination by oligodendrocytes (10, 12).

The mechanisms by which TMEV infection of the CNS induces demyelination are still not fully understood. Persistent infection of the CNS (13, 14) and the demonstration of viral RNA and antigen in oligodendrocytes during the chronic disease (15, 16) and demyelination in athymic nude mice (17, 18) support direct viral effects that can lead to demyelination. Other observations suggest that immune-mediated mechanisms are responsible for demyelination (19–24).

The complete nucleotide sequences of members of both TMEV subgroups (GD-VII vs. TO) have been determined (1, 3, 4), and infectious cDNA of representative strains of each subgroup have been prepared (25) (Roos, R. P., personal communication). It should now be possible to use a combination of sequence comparison and construction of recombinants between the strains to identify specific sequences that are responsible for the differences in pathogenesis in TMEV.

This approach has been successful in identifying sequences responsible for attenuation in the Sabin attenuated vaccine strains of poliovirus. Comparisons between the Sabin strain of type 3 poliovirus, its neurovirulent parent Leon, and a neovirulent revertant isolated from a fatal vaccine associated case of poliomyelitis, suggested that a substitution at position 472 in the 5′ noncoding region of the genome was an important determinant of attenuation in the P3/Sabin strain (26). Subsequent construction of genetic recombinants between the P3/Sabin and P3/Leon strains confirmed that the substitution at position 472 was an important marker of attenuation and identified a second attenuating mutation, a mutation resulting in the substitution of phenylalanine (Sabin) for serine (Leon) at amino acid 91 of the capsid protein VP-3 (27). Recombinants have also been used to show that mutations within the 5′ noncoding region of the Sabin strain of type 1 poliovirus contribute significantly to its attenuation and that a single 10-residue stretch of amino acids in the capsid protein VP-1 is an important determinant of host range in the mouse adapted Lansing strain of type 2 poliovirus (21, 28).

Although the use of sequence comparisons and genetic recombinants is expected to be valuable in identifying general regions of the genome responsible for differences in pathogenicity of Theiler's virus, the large number of sequence differences between naturally occurring strains presents a significant obstacle in identifying specific sequence differences that are responsible for differences in phenotype. We have, therefore, used an alternative approach, namely the characterization of variants with altered pathogenicity. mAbs have been used to select for variants of reovirus (29) and mouse hepatitis virus (30, 31) that were shown to be attenuated. Previously, we described an altered and diminished pattern of diseaese in immunocompetent SJL/J mice infected with such a variant virus (32). This variant virus (H7A6-2) was selected with a neutralizing mAb H7. The variant was shown to initiate less inflammatory lesions in the spinal cord and was cleared from the CNS. The observed changes in the pattern of disease may reflect how the immune system interacts with this determinant in the context of virus infection. Alternatively, modifications to structural proteins may effect the ability of the variant virus to infect oligodendrocytes and produce inflammation and concomitant demyelination.

In this report we have characterized the variant by investigating its ability to be bound and neutralized by mAbs as well as by sequencing selected regions of the RNA genome. These studies demonstrate that the variant contains an alteration in a neutralizing epitope located in the capsid protein VP-1, and suggest that a single point mutation at amino acid 101 of VP-1 is responsible for the attenuation of the variant. Model building based on sequence alignments and the known structure of the related Mengo virus (33) shows that the altered amino acid is located in an exposed loop on the surface of the virus at the periphery of a site that has been proposed to be the receptor binding site. Thus, the results of this study provide for the first time an opportunity to precisely map an important structural determinant of virally induced demyelination.

Materials and Methods

Cells. BHK-21 cells were cultured in DME (Cell Culture Facility, University of California, San Diego, CA), supplemented with 10% FCS (HyClone Laboratories, Logan, UT), nonessential amino acids, glutamine, sodium pyruvate, penicillin, streptomycin, and amphotericin B (Irvine Scientific, Santa Ana, CA). Cells were passaged twice weekly. BHK-21 cells were kindly provided by Drs. Don Summers and Ellie Ehrenfeld at the University of Utah, Salt Lake City, UT.

ELISA and Antibody (mAb). The ELISA was performed as described earlier by Rice and Fujinami (34). For viral antigen, cytoplasmic extracts from cells infected with either GD-VII, the H7A6-2 variant, or the parental DA viruses were used. In this study the mAbs used were mAb H5, a nonneutralizing antibody, and two neutralizing antibodies mAb H7, used to select the variant virus and mAb H8. All three mAbs recognize VP-1 of the DA strain by Western immunoblots and have been characterized in detail (35).

Virus and Variant Virus Selection. The DA strain of TMEV used in this study as the parental strain was originally obtained from J. Lehrich and B. Arnason (36). Passage history and variant virus selection have been previously described (32). Briefly, DA virus was incubated with neutralizing mAb to VP-1 for 1 h and then plaqued on BHK-21 cell monolayers. Plaques were picked and the procedure was repeated a total of three times. Variant viruses that "escaped" neutralization were additionally plaque purified and a stock of these variants was prepared in BHK-21 cells. A variant virus (H7A6-2), which showed a markedly reduced pathogenicity (32), was used for sequence analysis, as well as the parental DA virus.

The GD-VII virus strain used for antibody studies was purchased from American Type Tissue Collection (Rockville, MD) and was propagated similarly as the DA strain in BHK-21 cells. Antigen from this highly virulent strain was also used for ELISA (34).

Neutralization Assay. Approximately 200 plaque forming units (PFU) of variant virus (H7A6-2), parental DA, or GD-VII viruses in 100 μl were incubated with various dilutions of mAbs H5, H7, or H8 in 10 μl for 1 h on ice. The nonneutralizing mAb H5 served as a negative control. The virus-antibody solution was allowed to adsorb onto BHK-21 cell monolayers for 1.5 h at room temperature. The cell monolayers were overlaid with 0.5% agarose in medium 199 (Gibco Laboratories, Grand Island, NY), and incubated for 4 d at 37°C. After fixation and staining with 1% crystal violet, plaques were enumerated (37).

Polyacrylamide Gel Electrophoresis (PAGE). BHK-21 cells infected with the variant virus or the DA virus were harvested and processed for virus purification as described (35). The purified virus pellets were resuspended in SDS-sample preparation buffer (38) and analyzed on 14% polyacrylamide protein gels.

Preparation of Infected Cellular RNA Templates. Total cellular RNA was prepared from 3×10^8 suspension BHK-21 cells, grown in 150-cm^2 culture flasks (Costar, Cambridge, MA). These cells infected with either parental DA or with variant viruses were harvested 22 h after infection. Under these conditions both virses grew to similar titers as judged by previous plaquing studies. After washing with PBS the infected cells were resuspended in 1 ml of 25 mM sodium citrate pH 7.0 (Fisher Scientific, Pittsburgh, PA). The cells were then lysed by

the addition of 8 ml of GTC solution (4 M guanidinium thiocyanate from International Biotechnologies, New Haven, CT; 25 mM sodium citrate, 0.5% sodium lauryl sarcosine from Sigma Chemical Co., St. Louis, MO; 0.1 mM 2-ME from Fisher Scientific) and the DNA was sheared by vortexing. The lysate was then overlaid onto a 2 ml 5.7 M cesium chloride (Sigma Chemical Co.) cushion, and then centrifuged at 30,000 rpm in an SW41 rotor (Beckman Instruments, Fullerton, CA) at 15°C for 20 h. The resulting RNA pellet was dissolved in diethylpyrocarbonate (Behring Diagnostics, La Jolla, CA)-treated water and phenol/chloroform was extracted.

Oligonucleotide Primers. Selected oligonucleotide primers complementary to different regions of the published DA virus sequence (1) were synthesized on a gene assembler (Pharmacia Fine Chemicals, Uppsala, Sweden). After purification on a 16% sequencing gel, 50 pmol of oligonucleotides were endlabeled with γ-[^{32}P]ATP (Amersham Corp., Arlington Heights, IL) using T4-polynucleotide kinase (Boehringer Mannheim Biochemicals, Indianapolis, IN) in 50 mM Tris pH 7.5, 10 mM MgCl2, 5 mM dithiothreitol (DTT) (Sigma Chemical Co.), 0.1 mM spermidine, and 0.1 mM EDTA (Sigma Chemical Co.).

RNA Sequencing. A dideoxynucleotide RNA sequencing technique previously described (39) was used. Basically, 5 ng of ^{32}P-labeled oligonucleotide primer were mixed with 10 μg RNA in a total of 12 μl annealing buffer (50 mM Tris, pH 8.3, 60 mM NaCl, 10 mM DTT, and 1 mM EDTA). After heating at 90°C for 3 min the oligonucleotides were annealed to the RNA at 45°C for 30 min. Four reactions were carried out in wells of a 96-well plate labeled T, C, G, and A. To each well, 2 μl sequencing reaction mix (80 mM Tris pH 8.3, 100 mM NaCl, 15 mM DTT, 10 mM MgAc2, 1 mM of each dNTP and 0.1 mM of either ddTTP, or ddCTP, or ddGTP, or ddATP; Pharmacia LKB Biotechnology, Piscataway, NJ), 2 μl of the annealed oligonucleotide-RNA solution, 2 μl diluted AMV reverse transcriptase (3 U/well, Boehringer Mannheim Biochemicals) were added. The reaction was carried out at 45°C for 30 min followed by the addition of 2 μl of a chase solution (0.25 mM of each dNTP, 0.15 M KCl, 50 mM Tris pH 8.3, 10 mM MgCl2) and incubated for 15 min. The reaction was stopped with 4 μl formamide dye buffer (0.1% xylene cyanol, 0.1% bromophenol blue, 0.37% EDTA in deionized formamide; J. T. Baker Chemical Co., Phillipsburg, NJ). After boiling for 5 min the samples from the variant virus and parental DA virus were loaded in separate lanes onto a 6% acrylamide, 8 M urea sequencing gel and separated at 40 W for 1.5 h (39). After autoradiography the DNA sequence complementary to the viral RNA sequence from the variant virus, the parental DA virus and the earlier published DA virus sequence (1) were compared. In some instances formamide gels were used to unambiguously ascertain regions where stops and/or compressions were not fully resolved by the conventional gels. Briefly, 6% acrylamide, 8 M urea, and 40% deionized formamide sequencing gel were run at 40 W for 2.5 h (40). After drying the gels were processed for autoradiography.

Results

In a previous report we described a TMEV variant (H7A6-2) resistant to neutralization by a mAb, H7 (32). After infection of mice with this variant virus an altered and diminished disease pattern compared with the parental DA wild-type strain was observed. The variant virus produced significantly less inflammatory lesions and smaller numbers of infected cells were demonstrated with immunohistochemistry. The number of infected cells in the wild-type mice steadily increased, whereas the virus-positive cells from the variant mice gradually decreased after 1 wk after infection. Variant virus was cleared from the CNS, whereas wild-type virus persisted in the spinal cord. This variant virus was selected with a neutralizing mAb to VP-1. In this report we defined the exact sequence difference in VP-1 between the variant and parental DA virus, which is apparently responsible for neutralization escape and altered pathogenesis.

ELISA. The ability of several mAbs to bind the variant, the parental DA strain, and the neurovirulent GD-VII strain were examined by ELISA. ELISAs were per-

formed using two different neutralizing mAbs, H7 and H8, and a nonneutralizing mAb H5, which all have been shown to be directed to the capsid protein VP-1 (35). Cytoplasmic extracts prepared from BHK-21 cells infected with GD-VII (virulent), DA (TO type), or the variant H7A6-2 viruses served as antigens. mAb H5 bound to all three tested virus strains. By ELISA mAb H8 had a relative titer of 13.4 to DA virus, 13.4 to GD-VII virus, and 14.9 to H7A6-2 viral antigen (Table I). Thus mAb H8 reacted with all three viral strains. mAb H7, which was used to select the neutralization resistant variant H7A6-2, reacted only with viral antigen prepared from cells infected with the wild-type DA virus strain. mAb H7 bound neither to GD-VII nor to H7A6-2 virus variant. More over, mAb H7 does not recognize the BeAn strain of TMEV (Pevear, D. C., and H. L. Lipton, personal communication). These data indicate that mAb H7 recognizes a different epitope than mAb H8 and H5 and that epitopes recognized by H8 and H5 are unchanged in the variant. Furthermore, the results demonstrate that the variant virus H7A6-2 has been changed at an epitope in which GD-VII and BeAn virus differ from the wild type DA virus strain.

Neutralization Assay. The ELISA results indicated differences in the various mAbs ability to bind to various strains. To determine whether the change in the H7 binding epitope altered the ability of the mAbs to neutralize variant virus, versus parental DA and GD-VII viruses, neutralization assays were conducted. Binding of antibody does not necessarily reflect its ability to neutralize infectivity, mAb H7 neutralized only DA virus (Table II). mAb H5, the negative control that has the ability to bind to DA virus as determined by ELISA did not neutralize any of the three virus strains.

TABLE I

ELISA Reactivity: Lack of Binding of mAb H7 to Variant or GD-VII

	mAb*		
Virus antigen	H5	H7	H8
DA	14	21.7	13.4
H7A6-2	15.4	2.2	14.9
GD-VII	16.3	0	13.4

The ELISA was performed using mAb H5, H7, and H8 and cytoplasmic extracts from cells infected with either the parental DA, the GD-VII, or the variant H7A6-2 for viral antigen.

* Relative titer in $\log^2$

TABLE II

Lack of mAb H7 to Neutralize Variant or GD-VII

	mAb*		
Virus antigen	H5	H7	H8
DA	–	+	+
H7A6-2	–	–	+
GD-VII	–	–	+

Neutralization assays were performed with ~200 PFU of either parental DA, GD-VII, or variant H7A6-2 virus and various dilutions of mAbs H5, H7, or H8. Neutralization was scored as + when all infectivity was eliminated.

* At a dilution of 1/1,000 and comparable levels of immunoglobulins in H5, H7, and H8.

mAb H8 neutralized all three viruses, DA, GD-VII, and H7A6-2, indicating that mAb H8 and H7 define two different neutralizing epitopes within VP-1. In contrast to the epitope recognized by mAb H7 that appears to be highly specific for the DA strain, the epitope recognized by mAb H8 appears to be conserved in virus strains with grossly altered pathogenicity and is therefore unlikely to represent an area on the virion surface which is important for pathogenesis.

Migrational Characteristics. To determine if a portion of VP-1 from the variant virus H7A6-2 was deleted such as with coronaviruses (Parker, S., and M. J. Buchmeier, manuscript in preparation) or major changes in the migrational characteristics could be observed, the viral capsid proteins were analyzed by PAGE. No demonstrable differences in the migration of the capsid proteins, VP-1, VP-2, VP-3 and VP-4 were observed between DA and H7A6-2 virus (data not shown). Thus, there was no major deletion in the H7A6-2 variant virus proteins, particularly VP-1. These data support the antibody binding and neutralization studies (above) where major changes in binding were not observed i.e., changes at one site did not affect other sites.

RNA Sequencing. To precisely define the mutation(s) in the H7A6-2 variant, we sequenced selected regions of the viral RNA. Because ELISA and neutralization analysis demonstrated that mAb H7 binds to the wild-type DA strain but not to the H7A6-2 variant and recognizes VP-1 of the DA strain by Western blots, the entire coding region for VP-1 was sequenced. Two additional regions including 110 nucleotides in the 5′ noncoding region of the genome, and 105 nucleotides in the region of the genome encoding the capsid protein VP-2 were sequenced. Taken together the three regions sequenced account for ~15% of the total viral genome. Because our parental wild-type strain and the DA strain sequenced by Ohara et al. (1) have undergone multiple independent passages, we also determined the corresponding sequences in our wild-type strain.

The parental DA strain was shown to differ from the sequence published by Ohara et al. (1) at five nucleotide positions (Table III). Two of the sequence changes (the replacement of a cytosine by a guanine at nucleotide 3429 and the replacement of a cytosine by a thymine at nucleotide 3666) were silent. The remaining three substitutions resulted in the change of a serine to a threonine at amino acid 2 of the capsid protein VP-1 and the change of a leucine to a tryptophan at amino acid 214 of VP-1. Since the two wild-type DA strains cause a similar disease in SJL/J mice, it is unlikely that these changes are relevant to the pathogenesis of Theiler's virus.

TABLE III

Sequence Comparisons between Two DA Wild-type Strains

Nucleotide change			Amino acid change		
Position	DA*	Our DA	Position	DA*	Our DA
3008	G	C	2	Ser	Thr
3009	C	G			
3429	C	G		Silent	
3644	T	G	214	Leu	Trp
3666	C	T		Silent	

Nucleotide and resulting amino acid substitutions within VP-1 of the published DA (DA*) virus versus our parental DA strain.

* Previously published DA sequence (1).

Comparisons of the sequence in the region of the genome encoding VP-1 in the parental DA virus strain and the variant virus H7A6-2 showed only one single nucleotide difference at position 3305 (Table IV). In addition these data explain why mAb H7 does not react with BeAn or GD-VII (Table I). At this position a cytosine was replaced by a thymine (Fig. 1, resulting in the replacement of a threonine (wild-type) by an isoleucine (variant) at amino acid 101 of VP-1. Alignment of the DA virus VP-1 sequence with the atomic structure of Mengo virus (33) localized this amino acid modification in the second loop connecting the B and C strands of the β barrel of VP-1 (Fig. 2, *left panel*). Model building studies based on sequence alignments and the known structure of Mengo virus suggest that this loop is highly exposed on the surface of the virion (Fig. 2, *right panel*), and previous studies by others (41) have identified this loop as a potential antigenic site in the BeAn Strain of TMEV.

The location of the amino acid substitution on the virion surface, together with the observation that mAb H7 binds VP-1 in Western blots (35) for wild-type but not variant virus, strongly suggests that this mutation is responsible for neutralization escape. Because the mutation was selected under conditions that favor selection of single point mutations, it is also likely that this mutation is responsible for the previously characterized alteration in the ability of the variant to cause the chronic demyelinating disease. However, in order to investigate the possibility that additional mutations contribute to neutralization escape or attenuation of demyelination, two additional regions of the genome were sequenced. The first region encodes amino acids 140–175 of the capsid protein VP-2. Model building based on sequence alignments and the known structure of Mengo virus suggested that these amino acids constitute the loop connecting the D and E strand of the β barrel of VP-2. Moreover, the modeling suggests that in the virion this loop is sufficienty close to the C-D loop of VP-1 (including amino acid 101) to constitute a portion of a single antigenic site. The second area, nucleotides 410–520 in the 5′ noncoding region of the genome, was selected because previous studies (26) had shown that mutations in the 5′ noncoding region were important determinants of attenuation in poliovirus. In both

TABLE IV

Sequence Comparisons Among Different TMEV Strains Beginning at Nucleotide 3295 of the DA Virus

DA1*	TCC	GGC	GGT	ACC	ACC			AAT	TTT	CCA
	S	G	G	T	T			N	F	P
DA2‡	TCC	GGC	GGT	ACC	ACC			AAT	TTT	CCA
	S	G	G	T	T			N	F	P
H7A6-2	TCC	GGC	GGT	ATC	ACC			AAT	TTT	CCA
	S	G	G	I	T			N	F	P
BeAn	TCT	GGC	GGC	GTC	AAC	GGT	GCC	AAC	TTT	CCG
	S	G	G	V	N	G	A	N	F	P
GD-VII	TCT	GGT	GGC	GCC	AAT	GGT	GCC	AAC	TTC	CCA
	S	G	G	A	N	G	A	N	F	P

Comparison of different TMEV sequences encoding the area with the point mutation in the H7A6-2 variant.

* Published sequence (1).

‡ Sequence of our DA strain.

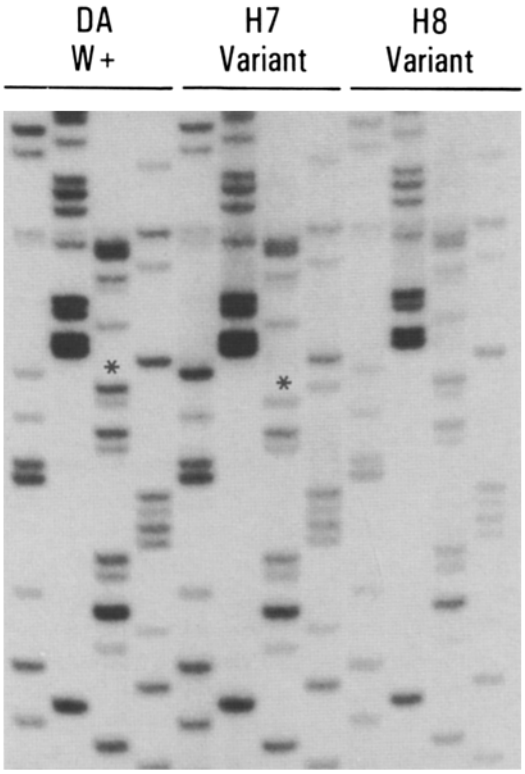

FIGURE 1. Sequence comparisons between the parental DA strain and the H7 variant showed only one single nucleotide difference at position 3305 (*). A cytosine (DA) was replaced by thymine (H7). The H8 variant showed no nucleotide differences at this area.

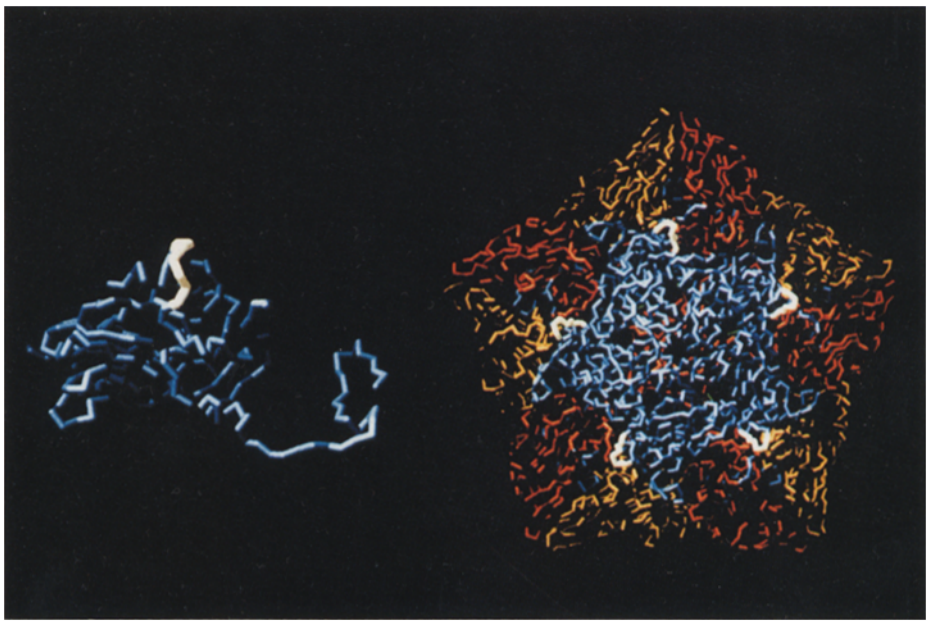

FIGURE 2. Alignment of the variant virus VP-1 sequence with the atomic structure of Mengo virus localized the modified amino acid on loop II of VP-1 (*left*) indicates that this loop is highly exposed on the surface of the virion (*right*).

of these regions the sequence of the H7A6-2 variant was identical to that of the parental DA strain.

Discussion

A variant virus of the DA-TMEV strain was selected by a neutralizing mAb. Infection of SJL mice with variant virus or parental wild-type virus resulted in major differences regarding size and number of inflammatory lesions and number of infected cells found in the CNS. The variant virus was less virulent than the wild-type virus and produced a markedly diminished disease (32). The purpose for this study was to molecularly define the precise mutation in the variant that is responsible for neutralization escape and attenuation by sequencing selected regions of the viral genome. Since the selecting mAb bound VP-1 from the wild-type but does not bind to the H7A6-2 variant at all, we focussed our sequencing efforts on the region of the genome coding for the capsid protein, VP-1. Two additional regions were sequenced, including the coding sequences for a region of VP-2, which has been proposed to be a component of a neutralizing epitope of Theiler's virus (41) and a portion of the 5′ noncoding region of the genome.

The variant was isolated under conditions that favor the selection of single point mutations. The variant and parental DA strains grow to similar titers and with very similar kinetics and titers in cell culture and in the brain early in infection (32), making it unlikely that the variant has an additional fortuitous mutation that affects its ability to replicate. Thus, the data suggest that the observed mutation is responsible for the attenuation of the demyelinating disease in the variant. Infectious transcripts of GD-VII (25) and of the DA strain (Roos, R. P., personal communication) have been constructed. They will allow genetic manipulations, e.g., single nucleotide replacements and evaluation of these changes on pathogenesis.

The parental DA strain used in these studies has been propagated separately from the DA strain sequenced by Ohara et al. (1) for at least 10 yr. Therefore, it was necessary to sequence our parental strain in parallel with the variant. Sequence comparisons between our DA strain and a published DA sequence (1) revealed five nucleotide differences. This lead to two amino acid changes within VP-1 and one nucleotide difference within the 5′ noncoding region. Even though these nucleotide differences exist between the DA virus of Roos and colleagues (17) and our DA wild-type virus (18) both DA viruses produce a similar disease. In contrast, the disease induced by variant virus H7A6-2 differs markedly from the pathology caused by the parental DA virus strain. The observed nucleotide differences in sequence between these two wild-type DA strains may be attributed to the high frequency of mutations within picornaviruses (42). Alternatively, at least some of the sequence differences may be ascribed to differences in sequencing techniques. Ohara et al. (1) used complimentary DNA as a template for their sequencing experiments. Our findings were generated from sequence analysis using viral RNA as templates. In both methods, the ability to sequence is dependent on transcription enzymes that have been shown to be error prone.

Comparison of the sequences of the H7A6-2 variant and our parental DA strain revealed only one nucleotide difference within the analyzed regions, namely, the substition of a thymine (in the variant) in place of cytosine (in the parent) at nucleotide 3305 (Fig. 1). This single nucleotide change led to a change in amino acid 101

of VP-1 in which threonine (in the parent) was replaced by an isoleucine (in the variant). No changes were found near the COOH-terminal trypsin cleavage site of VP-1, close to another neutralizing epitope (1), nor were there any sequence changes in the two other regions of the genome that were sequenced. Since this mutation is the only mutation found in VP-1, and since the mutation results in the loss of the ability of the selecting mAb H7 to bind to the variant virus, it is clear that this single mutation is responsible for neutralization escape. Consistent with the results of the binding and neutralization assays that show that the selecting mAb H7 recognizes only the parental DA strain, the mutation in the variant is located in a region of significant divergence in the known Theiler's virus sequences. Indeed, in addition to substantial sequence differences the GD-VII and BeAn strains contain two additional amino acids within this loop containing the mutation (Table IV).

The modified amino acid is located in the second of two loops connecting the C and D strands of the β barrel of VP-1. This loop (called loop II in the nonmenclature of Luo) (33) is highly exposed on the surface of the virion in a region previously identified as a potential neutralizing epitope in Theiler's virus (41). The loop is on the periphery of a deep depression (or "pit") in the surface of the virion that has been proposed as a probable receptor binding site in Mengo virus (33) and in Theiler's virus (41). This loop II region containing the observed amino acid difference in the variant virus H7A6-2 is of general importance for picornaviruses. In recombinant studies the same area determines the host range for poliovirus type 2 (28) and is important for neurovirulence of the Lansing strain type 2 in mice (21). Additionally, this structure in poliovirus types 1, 2, and 3 has been identified to contain a major neutralizing epitope (43). Peptides representing amino acids 90–104 of VP-1 inhibited neutralization of poliovirus type I by a neutralizing mAb. Antigenic poliovirus type 3 mutants resistant to neutralization by different mAbs had single point mutations clustered within this area (44, 45). The location of the mutation in the variant is consistent either with a mechanism in which the mutation alters the immune response to the virus in such a way that the variant is more efficiently cleared, or with a mechanism in which the mutation alters the ability to bind or infect the target cells for persistent infection (oligodendrocytes?) in the CNS. The question of how a mutation that destroys a neutralizing epitope in vitro causes an enhanced clearance in vivo, leads us to favor the second mechanism (alteration of cell tropism). Indeed, a similar mechanism has been used to explain the observation that sequence alterations in an exposed loop of VP-1 alter host range in poliovirus (21, 28).

This is the first demonstration of the epitope being important in the TMEV pathogenesis. The epitope was defined by ELISAs and neutralization assays using mAbs and the exact sequence of the epitope was determined. We describe here a single point mutation within the structural capsid protein VP-1, which alters the pathogenicity of the variant virus. The modified amino acid on loop II of VP-1 is part of a neutralizing epitope as determined by neutralization assays. This epitope has an important in vivo function in determining pathogenesis and neurovirulence.

Summary

Chronic Theiler's murine encephalomyelitis virus infection of susceptible mice is an animal model for human demyelinating diseases. Previously we described an altered and diminished pattern of central nervous system disease in immunocompe-

tent SJL/J mice infected with a variant virus (32). This variant virus H7A6-2 was selected with a neutralizing mAb recognizing the capsid protein VP-1 of Theiler's virus. Here we characterize the variant virus by ELISA and neutralization assays and by sequencing selected regions of the viral RNA genome and relate the alteration to disease. The variant virus contains one single point mutation within a neutralizing epitope of VP-1. This nucleotide change lead to an amino acid replacement at amino acid 101 of VP-1, a threonine (wild type) to an isoleucine (variant). Model building based on sequence alignments and the known structure of the related Mengo virus (33) indicates that the altered amino acid is located in an exposed loop on the surface of the virus at the periphery of a site that has been proposed to be the receptor binding site. The results of ELISA, neutralization assay, and direct RNA sequencing provide for the first time an opportunity to precisely map an important structural determinant of neurovirulence.

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