The 5' Noncoding Sequences from a Less Virulent Theiler's Virus Dramatically Attenuate GDVII Neurovirulence

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Received 21 February 1991/Accepted 15 May 1991

RNA transcripts derived from recombinant chimeras between the highly virulent GDVII virus and the less virulent BeAn virus were constructed to study the molecular pathogenesis of Theiler's murine encephalomyelitis virus infection. The presence of the BeAn 5' noncoding sequences in chimera 2 (BeAn 5' noncoding sequences joined with the GDVII nucleotides encoding the polyprotein and present in the 3' end) resulted in dramatic attenuation of GDVII neurovirulence and development of poliomyelitis in mice. This reduced neurovirulence was associated with slower virus growth and lower peak titers in the brain and spinal cord than with parental GDVII virus replication. On the other hand, the sites of replication following chimera 2 infection were the same as those seen in GDVII-infected mice; the distribution of virus antigen and histopathological changes indicated that chimera 2 replicates in neurons in the brain, e.g., in the neocortex, hippocampus, caudate putamen, and brain stem, as well as in anterior-horn cells in the spinal cord. Chimera 2 was efficiently cleared from the mouse central nervous system by day 30 postinfection, in marked contrast to the persistence of the BeAn parent in the central nervous system. This suggests that elements in the BeAn sequences that encode the polyprotein or are present in the 3' noncoding region are necessary for viral persistence. It is of interest that chimera 2-infected mice developed localized inflammatory, demyelinating lesions which were detected at day 28 postinfection but these lesions did not become larger with time. Thus, virus persistence appears to be required for maintenance and progression of immune-mediated demyelination. If the demyelinating lesions become sufficiently large, clinical signs and disease may develop.

Theiler's murine encephalomyelitis viruses (TMEVs) are naturally occurring enteric pathogens of mice which belong to the *Cardiovirus* genus of the family *Picornaviridae* (21, 23). TMEVs can be divided into two groups on the basis of their growth characteristics in vitro and neurovirulence after intracerebral (i.c.) inoculation (15). The first group consists of two highly virulent viruses, GDVII and FA, which produce rapidly fatal encephalitis in adult mice. All of the remaining isolates belong to a second group. These viruses are much less virulent but still cause central nervous system (CNS) disease in the form of poliomyelitis (early onset), followed by a chronic, inflammatory demyelinating disease (late onset) which is associated with viral persistence (14, 15). The difference in virulence between the two groups is substantial, on the order of $\geq 10^5$ PFU/50% lethal dose (LD_{50}) (16). The existence of two distinct neurovirulence groups makes TMEVs particularly useful for studying the molecular pathogenesis of picornaviruses.

Recently, we constructed full-length cDNAs of GDVII and BeAn virus RNAs and produced infectious transcripts in vitro (2). Progeny viruses had the in vitro growth phenotypes and mouse neurovirulence patterns of the respective parental virus strains. Analysis of recombinant chimeras constructed by exchanging corresponding genomic regions between parental cDNAs identified neurovirulence determinants in the 5' noncoding region and the P1 region, which encodes the viral coat proteins (2). Similar investigations conducted independently by two other groups have also mapped a neurovirulence determinant to the P1 region (9,

18). The presence of the BeAn 5' noncoding sequences in chimera 2 (BeAn 5' noncoding sequences joined with the GDVII nucleotides that encode the polyprotein and are present in the 3' end) resulted in dramatic attenuation of GDVII neurovirulence and development of poliomyelitis in mice (2). In addition, similar observations were made by Fu et al. (9), who found that the less virulent DA virus 5' noncoding sequences in their construct pGD1B-2C/DAFL3 attenuated GDVII neurovirulence but to a lesser extent. However, there are other differences between pGD1B-2C/DAFL3 and chimera 2.

We now report a detailed analysis of the pathogenesis of CNS infection and lower motor neuron disease in mice following i.c. inoculation of chimera 2. Temporal comparisons with infections following i.c. inoculation of mice with the parental viruses were made. The possible mechanism of TMEV attenuation and the relationship between TMEV persistence and demyelination are discussed.

MATERIALS AND METHODS

Cell culture. BHK-21 cells were grown in 100-mm-diameter plates in Dulbecco's modified Eagle medium (DMEM) supplemented with 2 mM L-glutamine, 0.15% tryptose phosphate broth (Sigma), 100 μ g of streptomycin, 100 U of penicillin, and 50 U of anti-pleuropneumoniae-like organism antibodies per ml (Gibco), and 10% iron-supplemented bovine calf serum (Hyclone).

Virus purification. BeAn 8386 virus was isolated from a feral mouse in Belem, Brazil, in 1957 and later classified as a TMEV by complement fixation serology (26). After plaque purification and amplification of the titer by several passages

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in BHK-21 cells, a working stock of BeAn virus was prepared. BeAn virus was purified by isopycnic centrifugation in Cs_2SO_4 as previously described (26).

Full-length cDNA TMEV clones and construction of the recombinant virus chimera 2. A full-length clone of BeAn virus was reconstructed from five subgenomic clones and cloned into the *Pst*I site of pBR322 (2). After mutation of an internal *Pst*I site at nucleotide 6694, the full-length clone was inserted into the *Pst*I site of transcription vector pGEM^R-3, downstream of the T7 RNA polymerase promoter. Similarly, a full-length GDVII virus cDNA clone was derived from six subgenomic fragments after initial cloning in pBR322 (7a). Recombinant virus chimera 2 was derived by exchanging the DNA sequences between the conserved restriction endonuclease site for *Ssp*I in the leader protein and the *Sca*I site in pGEM^R-3. The 10% difference in the nucleotide sequences of the two parental viruses (22) enabled confirmation of the origin of the exchanged genomic parts by cleavage with restriction endonucleases. In addition, chimera 2 was sequenced across the *Ssp*I site at the nucleotide 1138 joining site to confirm the presence of BeAn sequences. All plasmids were grown in *Escherichia coli* DH1 (10) and purified by equilibrium centrifugation in CsCl in the presence of ethidium bromide. DNAs were digested with restriction enzymes as directed by the manufacturers.

In vitro transcription. DNA clones oriented with the 5' genomic end downstream of the T7 promoter were linearized at the *Sph*I site within the vector downstream of the 3' end of the virus, and plus-strand RNA transcripts were synthesized. Reaction mixtures contained 40 mM Tris-HCl (pH 7.5), 6 mM MgCl_2 , 2 mM spermidine, 10 mM NaCl, each ribonucleoside triphosphate at 500 μM , 28 mM dithiothreitol, 50 U of RNasin RNase inhibitor (Promega), 2 to 4 μg of plasmid DNA, and 120 U of T7 RNA polymerase (Boehringer Mannheim) in 50 μl . Incubations were for 1 h at 37°C, and RNAs were analyzed for integrity and quantity on 0.6% native RNA agarose gels. The entire reaction mixture was used to transfect cells with or without prior DNase treatment.

Transfection. Subconfluent BHK-21 cell monolayers in 60-mm-diameter dishes were transfected with transcription reaction mixtures (approximately 10 μg of RNA) by using DEAE-dextran (29). Briefly, 0.5 mg of DEAE-dextran per ml in 400 μl of HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-buffered saline (137.5 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4 , 6 mM glucose, 20 mM HEPES, pH 7.0) was added to the transcription mixture and kept for 30 min at 4°C. BHK-21 cell monolayers were washed twice with DMEM, overlaid with the DEAE-dextran RNA mixture, incubated with gentle rocking for 40 min at 24°C, and overlaid with DMEM in which 2% Serum-Plus (Hazleton) and 1% bovine serum albumin (fraction V; Sigma) replaced the bovine calf serum. Cells were incubated at 33°C, and plaques appeared 3 to 5 days after transfection. A progeny virus stock of chimera 2 was prepared after an additional passage in BHK-21 cells.

Animal inoculations and determination of LD₅₀. SJL mice 4 to 6 weeks old were purchased from the Jackson Laboratory, Bar Harbor, Maine, and maintained on standard laboratory feed and water ad libitum in the University of Colorado Health Science Animal Care Center. Eight-week-old SJL mice were anesthetized with methoxyflurane and inoculated in the right cerebral hemisphere with 10-fold dilutions of parental or chimera 2 virus in 30 μl (five mice per dilution). Control mice received 30 μl of DMEM i.c. All animals were examined daily for the first 3 weeks and once

weekly thereafter for signs of encephalitis or the chronic gait abnormality indicative of demyelination. The mean LD₅₀ was calculated by the method of Reed and Muench (24).

Virus plaque assay. CNS tissues were assayed for virus titer by using a standard plaque assay (26). Brains and spinal cords were disrupted by sonication for two 30-s bursts at 4°C on setting 2 (15 W) of a Branson Sonifier and clarified by centrifugation for 10 min at 1,000 $\times g$ at 4°C. Dilutions of clarified samples were adsorbed to BHK-21 cells in 60-mm plastic dishes for 30 min at 24°C, and the cells were washed once in DMEM and overlaid with 5 ml of complete DMEM with 0.1% bovine serum albumin and 0.9% Noble agar (Difco, Detroit, Mich.). Cells were incubated for 4 days at 33°C, and plaques were identified by staining with 0.12% crystal violet.

Total RNA isolation. Mice were killed by exsanguination under methoxyflurane anesthesia, and their spinal cords were expelled from the spinal canals by perfusion with ice-cold phosphate-buffered saline. Total cellular RNA was isolated essentially by the method of Chomczynski and Sacchi (3). Typically 60 to 90 μg of total RNA was recovered from each spinal cord (wet weight, approximately 150 mg). The RNA was analyzed by electrophoresis in a 1% agarose gel containing formaldehyde (17), and on the basis of the integrity of the rRNA bands, the isolated RNA was considered intact.

Amplification of cDNA synthesized from TMEV RNA. The polymerase chain reaction (PCR) was performed with a Perkin-Elmer-Cetus DNA Thermal Cycler. Approximately 10 μg of total cellular RNA was used to synthesize cDNA by using primer 2 (described below) and avian myeloblastosis virus reverse transcriptase (Seikagaku America Inc.) in 25 μl . Five microliters of the cDNA reaction product from uninfected or chimera 2-infected mice or 0.5 μl of that from BeAn-infected samples was added to the amplification reaction. The final reaction mixture contained 10 mM Tris (pH 8.4), 50 mM KCl, 1.5 mM MgCl_2 , 0.01% (wt/vol) gelatin, deoxynucleoside triphosphates at 200 μM , each primer at 0.5 μM , and 2.5 U of *Taq* polymerase (Perkin-Elmer-Cetus) in 50 μl . Samples were subject to 30 cycles of amplification: 2 min at 94°C for denaturation, 2 min at 50°C for primer annealing, and 3 min at 72°C for polymerization, with successive cycles of polymerization increased by 15 s. Negative control amplifications were done by using 5 μl of the cDNA synthesis reaction product containing no RNA and also PCR reagents alone; amplification of 1 ng of cloned full-length BeAn virus cDNA was used as a positive control. Five microliters of the PCR product was electrophoresed in a 1% agarose gel and analyzed by Southern blot hybridization with a ³²P-labeled internal probe, 5' T(ACG)(AT)CG (CT)GAAG(AG)AAGCAGTTC 3', which corresponds to the BeAn virus sequence from nucleotides 686 to 705. The sequence of primer 1, which corresponds to BeAn nucleotides 527 to 546, was 5' GG(CT)C(GT)AAGCCGCT(CT)GG AATA 3'; the sequence of primer 2, which is complementary to BeAn nucleotides 809 to 790, was 5' CACGTGGCTTTTG GCCGCAG 3'.

Histology. Anesthetized mice were killed by perfusion with either 4% paraformaldehyde or 3% glutaraldehyde in phosphate buffer, pH 7.3, at the indicated times postinfection (two or three mice for each virus on days 3 to 8 postinfection). Brains and spinal cords from paraformaldehyde-perfused mice were embedded in paraffin, cut at 5 μm , and stained with hematoxylin and eosin. Spinal cords from glutaraldehyde-perfused mice on days 28, 49, and 63 postinfection were postfixed in 1% OsO_4 , dehydrated, and embed-

FIG. 1. Genomic representation of the BeAn and GDVII viruses and the chimera 2 (Chi 2) recombinant virus. (A) The location of the authentic AUG codon (nucleotide 1069) in the GDVII polypeptide (the AUG is at nucleotide 1064 in BeAn) is shown, as well as the restriction endonuclease *SspI* site at which the parental sequences were combined (nucleotide 1137). (B) The leader protein amino acid sequence for the parental viruses from the N terminus to the *SspI* site is shown; the single-residue difference is boxed.

ded in Epon as previously described (6); 1- μ m sections were stained with 1% toluidine blue.

Fluorescent-antibody staining. Brains from mice were quick-frozen, cut into 6- μ m axial sections in a cryostat, fixed in acetone for 10 min, and stored at -20°C . Indirect immunofluorescence staining for TMEV antigens and the specificity of the reagents were as previously described (15).

Measurement of virus-specific DTH responses. At indicated times postinfection, the ears of infected and uninfected (negative control) mice were measured with a Mitutoyo 7326 micrometer. Immediately thereafter, the dorsal side of each ear was injected with purified UV-inactivated BeAn virus or vesicular stomatitis virus (5 μ g in 10 μ l of phosphate-buffered saline) to elicit virus-specific delayed-type hypersensitivity (DTH) responses (4). At 24 h after challenge, each ear was again measured to determine the increase in thickness (swelling due to mononuclear cell infiltration); the increase was expressed in 10^{-4} in. (1 in. = 2.54 cm) units.

RESULTS

Construction and properties of recombinant virus chimera 2. Chimera 2 was derived by joining DNA representing the less virulent BeAn virus 5' end to DNA containing the sequences of the highly virulent GDVII virus by using the conserved site for restriction endonuclease *SspI* in the nucleotide sequences that encode the leader protein. In addition to the BeAn 5' noncoding region, nucleotides 1065 to 1138, which encode the initiation AUG codon and the N-terminal 24 amino acids of the BeAn leader protein, are contained in chimera 2 (Fig. 1A and B). The chimera 2 polypeptide differs from that of GDVII by only leader amino acid 22, which is glycine in BeAn and aspartic acid in GDVII. Like the highly virulent GDVII parent (2), chimera 2 formed large plaques upon growth in BHK-21 cells and was not temperature sensitive. Moreover, chimera 2 and GDVII showed similar one-step growth patterns (data not shown). However, chimera 2 showed attenuated neurovirulence compared with GDVII virus following i.c. inoculation of mice; the LD_{50} s were $>10^6$ and <5 PFU, respectively (Fig. 2).

Acute clinical disease. In separate experiments, 50 to 80% of adult SJL mice inoculated i.c. with 1×10^6 to 3×10^6 PFU of chimera 2 developed paralysis between 1 and 10 days

FIG. 2. Comparative neurovirulence of transcript-derived parental and chimera 2 (Chi-2) viruses after i.c. inoculation of mice. Adult SJL/J mice were inoculated i.c. with 1:10 virus dilutions in 30 μ l and observed daily for signs of illness and death. For BeAn and chimera 2, the LD_{50} endpoint in PFU was not reached.

postinfection (Table 1 and Fig. 3). The clinical disease was typical of the poliomyelitis seen following infection of mice with brain-derived less virulent strains of TMEV in which flaccid paralysis is localized mainly in the hind limbs (15). In these experiments, 10% of the chimera 2-inoculated mice died and the surviving animals often recovered from paralysis. Between days 2 and 5 postinfection, 100% of the mice inoculated with similar concentrations of GDVII virus developed signs of encephalitis and hind limb paralysis, and death occurred by 5 to 6 days postinfection. None of the mice inoculated with BeAn virus mice showed signs of clinical disease.

CNS viral growth. Following i.c. inoculation, parental-virus-inoculated and chimera 2 virus-inoculated mice were sacrificed at regular intervals, their brains and spinal cords were removed, and specimens from three mice at each time were pooled for viral titrations (Fig. 4a and b). The virus

TABLE 1. Incidence of paralysis and death in adult SJL/J mice inoculated i.c. with parental and chimera 2 viruses derived from RNA transcripts

Expt no.	Virus	Dose (PFU, 10^6)	No. with paralysis/total (%) ^a	No. dead/total (%) ^a
1	BeAn	2.9	0/10 (0)	0/10 (0)
	Chimera 2	2.9	8/10 (80)	1/10 (10)
2	GDVII	4.2	10/10 (100)	10/10 (100)
	BeAn	3.0	0/5 (0)	0/5 (0)
	Chimera 2	3.3	5/9 (55)	1/10 (10)
3	GDVII	1.0	10/10 (100)	10/10 (100)
	BeAn	1.0	0/10 (0)	0/10 (0)
	Chimera 2	1.0	5/10 (50)	1/10 (10)

^a Paralysis and mortality were recorded during days 1 to 21 postinfection.

FIG. 3. Time of onset of flaccid limb paralysis in SJL/J mice inoculated i.c. with approximately 10^6 PFU of transcript-derived GDVII (●), BeAn (■), and chimera 2 (△) viruses. During the first 6 days postinfection (PI), all mice were observed every 12 h for development of paralysis. In this experiment, 10 mice were included in each group and all of the GDVII virus-inoculated mice died by day 6 postinfection.

contents of brains and spinal cords of GDVII-infected mice reached peak titers of approximately 10^8 PFU/g by day 3 postinfection and then declined before death. The virus contents of brains and spinal cords of BeAn virus-infected mice reached a maximum of 10^5 PFU/g by day 4 postinfection, and subsequently the titer in brains declined to 10^3 PFU/g by day 14 postinfection. BeAn virus contents in spinal cords declined modestly after day 12 postinfection but remained at approximately 10^3 PFU/g on days 30 (Fig. 1B) and 65 (data not shown) postinfection. In mice inoculated with chimera 2, virus titers in brains reached a maximum of 10^6 to 10^7 PFU/g at day 5 postinfection, slightly later than

that observed for either parental virus and at a level similar to that observed for BeAn virus. Chimera 2 was cleared from spinal cords between days 15 and 30 postinfection (Fig. 1B), and no infectious virus was detectable on day 65 postinfection (data not shown). These data suggest that the nucleotides that encode the polyprotein and/or the 3' noncoding sequences of the less virulent BeAn virus are required for viral persistence.

Absence of chimera 2 sequences during the chronic phase of infection. Although infectious virus was not detected in spinal cords of chimera 2 virus-infected mice on days 30 and 65 postinfection, the plaque assay used has a limit of detection of 50 PFU/g of tissue. Therefore, we amplified cDNA synthesized from total cell RNA isolated from spinal cords of mice infected with either BeAn or chimera 2 by using a primer complementary to genomic 5' noncoding sequences. A DNA fragment of the expected size, 283 bp, was amplified in samples processed from total RNA obtained from a BeAn virus-infected mouse at day 150 postinfection, whereas none was observed in three chimera 2-infected mice sacrificed at the same time point (Fig. 5). By PCR, we were consistently able to detect as little as 1 fg of virus RNA in the total RNA isolated from spinal cords of BeAn-infected mice, even as late as day 330 postinfection (data not shown).

Acute pathological CNS changes and distribution of viral antigen(s). There were no gross abnormalities of the brains or spinal cords of mice inoculated with parental or chimera 2 virus. Parenchymal involvement was seen between days 3 and 8 postinfection, principally in the gray matter of the cerebral neocortex, caudate putamen, thalamus, hippocampus, brain stem, and spinal cord in both GDVII- and chimera 2-infected mice. The changes, consisting of chromatolysis, neuronophagia, microglial proliferation, and mononuclear cell infiltration, especially in perivascular sites, were more severe in GDVII- than chimera 2-infected mice. Small numbers of mononuclear inflammatory cells were also present in the leptomeninges. Inclusions were not observed. Neocorti-

FIG. 4. Growth of virus in brains (a) and spinal cords (b) of SJL/J mice inoculated i.c. with approximately 10^6 PFU of transcript-derived GDVII (●), BeAn (■), and chimera 2 (△) viruses. Each symbol represents three pooled brains or spinal cords at each time point. The limit of virus detection was 50 PFU. Note that all GDVII virus-inoculated mice died by day 6 postinfection (PI).

FIG. 5. Southern blot of cDNA derived from spinal cords of BeAn- or chimera 2-infected mice. PCR-amplified cDNA reverse transcribed with a primer complementary to the BeAn sequence from 790 to 809 and total cellular RNA isolated from cell culture or spinal cords were used. Lanes: 1, PCR reagents only; 2, control cDNA reaction in the absence of RNA; 3, BeAn virus-infected mouse; 4, BHK-21 cells infected with BeAn virus; 5, uninfected control mouse; 6 to 8, three chimera 2-infected mice. The amplification procedure is described in Materials and Methods. The primers coincide with the BeAn sequence from 527 to 546 and the complementary sequence from 790 to 809. The probe which was complementary to the BeAn virus sequence from 686 to 705 was end labeled with [γ^{32} P]ATP. An expected 283-bp band was amplified and detected by using this probe from infected BHK-21 cell RNA (lane 6). ori, origin of gel.

cal changes were largely restricted to the inferior frontal and occipital regions, with involvement mainly of the lower layers of the neocortex (Fig. 6A and B). In GDVII virus-infected mice, necrosis of the pyramidal cell layer of the hippocampus with accompanying polymorphonuclear leukocyte infiltration (Fig. 6D) and of patchy areas of the brain stem was observed by day 3 postinfection. Necrotizing lesions were not seen in mice inoculated with chimera 2 (Fig. 6E), except for the occasional moribund animal, in which the changes were as severe as in GDVII virus-infected mice. In both GDVII- and chimera 2-infected mice between days 3 and 7 postinfection, viral antigen(s) was detected in clusters of cells in the areas of pathological involvement in the neocortex, the pyramidal cell layer of the hippocampus, the thalamus, and the brain stem (data not shown). More viral antigen-positive cells were found in GDVII-infected mice than in chimera 2-infected mice. Unlike the severe changes seen in mice inoculated with GDVII virus, BeAn virus-inoculated mice showed only minimal pathological changes in the hippocampus and brain stem, and in many animals no histopathological changes were observed (Fig. 6C and F).

Chronic demyelination. Spinal cords of mice inoculated i.c. with parental BeAn or chimera 2 virus were examined at days 28 and 49 or 63 postinfection for the inflammatory, demyelinating lesions associated with BeAn virus persistence. On day 28 postinfection, small patchy areas containing mononuclear cell infiltrates and some naked axons were found in chimera 2-infected mice. However, there was no progression of such lesions on day 63 postinfection (Fig. 7A), indicating that infection with chimera 2 resulted in demyelination during the first month postinfection but did not lead to chronic, progressive demyelination. On the other hand, BeAn virus-infected mice showed extensive mononu-

clear inflammation and demyelination, including influx of lipid-laden macrophages (Fig. 7B), as previously described (5).

Time course of TMEV-specific DTH responses. Since chronic high levels of virus-specific DTH are characteristic of BeAn virus-induced demyelinating disease and are presumably due to ongoing antigenic virus stimulation (4), virus-specific DTH was assayed in mice inoculated i.c. with UV-inactivated BeAn virions. BeAn virus cellular immune responses (T-cell proliferation and DTH responses) cross-react with GDVII virus (19). As shown in Fig. 8, infection with BeAn or chimera 2 virus was accompanied by significant levels of virus-specific DTH, beginning by day 7 postinfection and continuing to day 65 postinfection. However, compared with BeAn virus-infected mice, virus-specific DTH was not sustained in chimera 2-infected mice after day 65 postinfection, suggesting that persistent infection is required to maintain antigenic stimulation and chronic virus-specific DTH responsiveness in TMEV infection.

DISCUSSION

This report describes the properties of a recombinant TMEV which contains the less virulent BeAn genomic 5' noncoding region joined to the highly virulent GDVII sequences that encode the polyprotein and the 3' noncoding end. In mice, this recombinant, chimera-2, showed markedly attenuated neurovirulence compared with GDVII. This attenuation in PFU per LD₅₀ was substantial, on the order of $\geq 10^5$. Until now, mice inoculated i.c. with GDVII either died or did not become infected; lack of infection in survivors was based on absence of an anti-TMEV antibody response (16). Use of chimera 2 resulted in 50% survival after i.c. inoculation, even at a dose of 10^6 PFU. This reduced neurovirulence was associated with slower virus growth and lower peak titers in brains and spinal cords than with parental GDVII virus replication. On the other hand, the sites of replication following chimera 2 infection were the same as those seen in GDVII-infected mice; the distribution of virus antigen and histopathological changes indicated that chimera 2 replicates in neurons in the brain, e.g., in the neocortex, hippocampus, caudate putamen, and brain stem, as well as in anterior-horn cells in the spinal cord. BeAn virus antigen was not detected in neocortical neurons but was observed in the other CNS sites. Survival of chimera 2-infected mice is associated with reduced virus growth in the brain, enabling clinical manifestation of the flaccid paralysis reflective of anterior-horn cell destruction. We are unable to explain why virus titers in the spinal cords of chimera 2-infected mice were no higher than those observed in BeAn-infected mice which did not develop early flaccid paralysis.

Chimera 2, which contains sequences that encode the GDVII polyprotein, in fact, has one amino acid of BeAn, a glycine for aspartic acid at position 22 of the leader protein. The leader protein, which is unique to cardioviruses and aphthoviruses in the family *Picornaviridae*, is 76 amino acids long; no known function has been discovered for the cardiovirus leader protein. DA virus, the only other less virulent TMEV which has been sequenced, also has a glycine at position 22. Without site-specific mutation of this codon, one cannot exclude an attenuating effect of glycine at this position in the leader protein. However, we have found that another recombinant virus which contains nucleotides 1 to 933 of BeAn virus joined with the sequences of GDVII virus [934-poly(A) tail] is almost as attenuating as chimera 2

FIG. 6. Paraffin-embedded axial sections of brains from mice inoculated i.c. and killed on days 3 to 5 postinfection, stained with hematoxylin and eosin. Occipital lobe sections at a magnification of $\times 100$ are shown in panels A to C. Panels: A, GDVII-infected mouse showing neuronophagia, perivascular infiltration, and mononuclear cell infiltrates in the lower layers of the cortex; B, chimera 2-infected mouse showing a small amount of neuronophagia and mononuclear cell infiltrates; C, BeAn-infected mouse showing normal histology. Hippocampal pyramidal cell layers at a magnification of $\times 400$ are shown in panels D to F. Panels: D, GDVII-infected mouse showing necrosis and a few polymorphonuclear leukocytes; E, chimera 2-infected mouse showing neurons undergoing degeneration and mononuclear cell infiltration; F, BeAn-infected mouse showing essentially normal histology.

(LD₅₀, 2×10^5 PFU; 23a). This suggests that the major and perhaps only determinant of attenuation in chimera 2 is in the 5' noncoding region.

The attenuation of neurovirulence by sequences in the 5' noncoding region of a less virulent TMEV may be analogous to reports of the involvement of the 5' noncoding sequences of the human polioviruses in determining neurovirulence. In the most extensively studied of the three serotypes, poliovirus type 3, a C→U transition at position 472 results in attenuation and, conversely, a U→C transition is associated with restored neurovirulence (12, 30). Mutations at position 480 in the 5' noncoding region of type 1 poliovirus and position 481 of type 2 poliovirus appear to have similar effects on neurovirulence (20, 25). Considerable evidence

suggests that these mutations selectively affect the efficiency of poliovirus translation in neural cells (1, 12, 13, 27, 28). Mutations at 472, 480, and 481 may alter the way in which ribosomes and/or RNA-binding proteins interact with internal sequences that constitute the ribosome landing pad (reviewed in reference 11). A major determinant of poliovirus neurovirulence may therefore involve a selective block in viral translation in anterior-horn cells. Similarly, in TMEVs, interaction between the 5' noncoding sequences of GDVII and BeAn and a host protein(s) may also play a role in modulating the course of infection in mice.

Recently, Duke et al. (7) showed that in mengovirus, another cardiovirus, truncation of the homopolymeric poly(C) tract in the 5' noncoding sequences dramatically attenuates

FIG. 7. Representative spinal cord sections from mice inoculated i.c. and killed at approximately 2 months postinfection. The 1- μ m-thick Epon-embedded sections stained with toluidine blue reveal a small demyelinating lesion in close proximity to meningeal mononuclear inflammatory cells in the anterior column of a chimera 2-infected mouse killed on day 63 postinfection (magnification, $\times 220$) (A) and an extensive demyelinating lesion with mononuclear inflammatory cells and lipid-laden macrophages in the anterior column of a BeAn virus-infected mouse killed on day 49 postinfection (magnification, $\times 220$) (B).

neurovirulence, also indicating a role for this region in determining the severity of infection. The mechanism for poly(C)-mediated attenuation, however, is unknown.

Chimera 2 was efficiently cleared from the mouse CNS by day 30 postinfection, in marked contrast to the persistence of the BeAn parent in the CNS. This suggests that elements in the BeAn sequences that encode the polyprotein or are present in the 3' noncoding region are necessary for viral persistence. This notion is consistent with a recent study using the technique of *in situ* hybridization in which a chimeric TMEV containing the P1 sequences of GDVII that encode the coat proteins joined to the less virulent DA virus reportedly did not persist in the CNS white matter, whereas a recombinant in which only the VP1 sequences of DA virus

FIG. 8. Temporal pattern of virus-specific DTH responses in SJL/J mice inoculated i.c. with approximately 10^6 PFU of either BeAn or chimera 2 (X-2) virus. Each group consisted of two to four mice. The results are expressed as ear-swelling responses at 24 h (background responses of negative control mice subtracted) in units of 10^{-4} in. $\pm$ the standard error of the mean (SEM). Only background responses were observed in BeAn and chimera 2 virus-infected mice challenged with 5 μ g of purified vesicular stomatitis virus (unrelated-virus control) on days 28 and 65 postinfection (data not shown).

were inserted into a full-length GDVII clone persisted (18). Thus, sequences in the less virulent TMEV may be required for persistent infection of the CNS, and the critical region for viral persistence includes the sequences that encode VP1.

The results of pathogenetic studies of chimeric-TMEV-infected mice provide insight into the mechanism of TMEV-induced demyelination. Recently, Fu et al. (8) described demyelination at day 45 postinfection in susceptible SJL mice inoculated with a TMEV recombinant containing GDVII sequences for most of P1 (C termini of VP2, VP3, and VP1) and P2 (proteins 2A and 2B and the N terminus of 2C). They did not report whether the demyelinating lesions progressed over time. In contrast, when inoculated with this recombinant virus, the susceptible mouse strain DBA/2 did not develop demyelination, perhaps indicating a reduced ability of this recombinant to demyelinate (8). In the present study, chimera 2-inoculated mice did not sustain levels of virus-specific DTH as high as those of mice inoculated with the BeAn parent. Previously, we provided strong support for a causative role of TMEV-specific DTH in demyelination (4). However, chimera 2-infected mice developed localized inflammatory, demyelinating lesions which were detected at day 28 postinfection; however, the lesions did not become larger with time. Therefore, it appears that elements within the GDVII genome may initiate myelin breakdown. Thus, TMEV persistence appears to be required for maintenance and progression of demyelination, which is immune system mediated, and development of clinical disease (if lesions become sufficiently large).

ACKNOWLEDGMENTS

This research was supported by U.S. Public Health Service grants NS 21913, NS 23349, and NS 13011 from the National Institutes of Health, National Multiple Sclerosis Society grant RG 1815 B-7, and funds from the Leiper Foundation.

REFERENCES

1. Agol, V. I., S. G. Drozdov, T. A. Ivannikova, M. S. Kolesnikova, M. B. Korolev, and E. A. Tolskaya. 1989. Restricted growth of

- attenuated poliovirus strains in cultured cells of human neuroblastoma. *J. Virol.* **63**:4034–4038.
2. Calenoff, M. A., K. S. Faaberg, and H. L. Lipton. 1990. Genomic regions of neurovirulence and attenuation in Theiler's murine encephalomyelitis virus. *Proc. Natl. Acad. Sci. USA* **87**:978–982.
 3. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**:156–159.
 4. Clatch, R. J., H. L. Lipton, and S. D. Miller. 1986. Characterization of Theiler's murine encephalomyelitis virus (TMEV)-specific delayed-type hypersensitivity responses in TMEV-induced demyelinating disease: correlation with clinical signs. *J. Immunol.* **136**:920–927.
 5. Dal Canto, M. C., and H. L. Lipton. 1975. Primary demyelination in Theiler's virus infection. An ultrastructural study. *Lab. Invest.* **33**:626–637.
 6. Dal Canto, M. C., and H. L. Lipton. 1982. Ultrastructural immunohistochemical localization of virus in acute and chronic demyelinating Theiler's virus infection. *Am. J. Pathol.* **106**:20–29.
 7. Duke, G. M., J. E. Osorio, and A. C. Palmenberg. 1990. Attenuation of Mengo virus through genetic engineering of the 5' noncoding poly(C) tract. *Nature (London)* **343**:474–476.
 - 7a. Faaberg, K. S., et al. Unpublished data.
 8. Fu, J., M. Rodriguez, and R. P. Roos. 1990. Strains from both Theiler's virus subgroups encode a determinant for demyelination. *J. Virol.* **64**:6345–6348.
 9. Fu, J., S. Stein, L. Rosenstein, T. Bodwell, M. Routbort, B. L. Semler, and R. P. Roos. 1990. Neurovirulence determinants of genetically engineered Theiler viruses. *Proc. Natl. Acad. Sci. USA* **87**:4125–4129.
 10. Hanahan, D. 1983. Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.* **166**:557–580.
 11. Jackson, R. J., M. T. Howell, and A. Kaminski. 1991. The novel mechanism of initiation of picornavirus RNA translation. *Trends Biol. Sci.* **15**:477–482.
 12. LaMonica, N., J. W. Almond, and V. R. Racaniello. 1987. A mouse model for poliovirus neurovirulence identifies mutations that attenuate the virus for humans. *J. Virol.* **61**:2917–2920.
 13. LaMonica, N., and V. R. Racaniello. 1989. Differences in replication of attenuated and neurovirulent polioviruses in human neuroblastoma cell line SH-SY5Y. *J. Virol.* **63**:2357–2360.
 14. Lehrich, J. R., B. G. W. Arnason, and F. H. Hochberg. 1976. Demyelinating myelopathy in mice induced by the DA virus. *J. Neurol. Sci.* **29**:149–160.
 15. Lipton, H. L. 1975. Theiler's virus infection in mice: an unusual biphasic disease process leading to demyelination. *Infect. Immun.* **11**:1147–1155.
 16. Lipton, H. L. 1980. Persistent Theiler's murine encephalomyelitis virus infection in mice depends on plaque size. *J. Gen. Virol.* **46**:169–176.
 17. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual, p. 202–203. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
 18. McAllister, A., F. Tangy, C. Aubert, and M. Brahic. 1990. Genetic mapping of the ability of Theiler's virus to persist and demyelinate. *J. Virol.* **64**:4252–4257.
 19. Miller, S. D., R. J. Clatch, D. C. Pevear, J. L. Trotter, and H. L. Lipton. 1987. Class II-restricted T cell responses in Theiler's murine encephalomyelitis virus (TMEV)-induced demyelinating disease. *J. Immunol.* **138**:3776–3784.
 20. Nomoto, A., M. Kohara, S. Kuge, N. Kawamura, M. Arita, T. Komatsu, S. Abe, B. L. Semler, E. Wimmer, and H. Itoh. 1987. Study on virulence of poliovirus type 1 using in vitro modified viruses, p. 437–452. *In* M. A. Brinton and R. R. Rueckert (ed.), *Positive strand RNA viruses*. Alan R. Liss, Inc., New York.
 21. Ohara, Y., S. Stein, J. Fu, L. Stillman, L. Klamman, and R. P. Roos. 1988. Molecular cloning and sequence determination of DA strain of Theiler's murine encephalomyelitis viruses. *Virology* **164**:245–255.
 22. Pevear, D. C., J. Borkowski, M. Calenoff, B. Ostrowski, and H. L. Lipton. 1988. Insights into Theiler's virus neurovirulence based on a genomic comparison of the neurovirulent GDVII and less virulent BeAn strains. *Virology* **165**:1–12.
 23. Pevear, D. C., M. Calenoff, E. Rozhon, and H. L. Lipton. 1987. Analysis of the complete nucleotide sequence of the picornavirus Theiler's murine encephalomyelitis virus indicates that it is closely related to cardioviruses. *J. Virol.* **61**:1507–1516.
 - 23a. Pritchard, A., M. Calenoff, and H. L. Lipton. Unpublished data.
 24. Reed, L. J., and H. A. Muench. 1938. A simple method of estimating fifty percent endpoints. *Am. J. Hyg.* **27**:493–497.
 25. Ren, R., E. G. Moss, and V. R. Racaniello. 1991. Identification of two determinants that attenuate vaccine-related type 2 poliovirus. *J. Virol.* **65**:1377–1382.
 26. Rozhon, E. J., J. D. Kratochvil, and H. L. Lipton. 1983. Analysis of genetic variation in Theiler's virus during persistent infection in the mouse central nervous system. *Virology* **128**:16–32.
 27. Svitkin, Y. V., N. Cammack, P. D. Minor, and J. W. Almond. 1990. Translation deficiency of the Sabin type 3 poliovirus genome: association with an attenuating mutation C₄₇₂-U. *Virology* **175**:103–109.
 28. Svitkin, Y. V., S. V. Masolva, and V. I. Agol. 1985. The genomes of attenuated and virulent poliovirus strains differ in their in vitro translation efficiency. *Virology* **147**:243–252.
 29. Vaheri, A., and J. S. Pagano. 1965. Infectious poliovirus RNA: a sensitive method of assay. *Virology* **27**:434–436.
 30. Westrop, G. D., D. M. A. Evans, P. D. Minor, D. Magrath, G. C. Schild, and J. W. Almond. 1987. Investigation of the molecular basis of attenuation in the Sabin type 3 vaccine using novel recombinant polioviruses constructed from infectious cDNA, p. 53–60. *In* D. J. Rowlands, M. A. Mayo, and B. W. J. Mahy (ed.), *The molecular biology of the positive strand RNA viruses*. Alan R. Liss, Inc., New York.