

Attenuation of Venezuelan Equine Encephalitis Virus Strain TC-83 Is Encoded by the 5'-Noncoding Region and the E2 Envelope Glycoprotein

RICHARD M. KINNEY*, GWONG-JEN CHANG, KIYOTAKA R. TSUCHIYA, JUDITH M. SNEIDER, JOHN T. ROEHRIG, TONJA M. WOODWARD, AND DENNIS W. TRENT

Division of Vector-Borne Infectious Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Fort Collins, Colorado 80522-2087

Received 18 August 1992/Accepted 10 November 1992

The virulent Trinidad donkey (TRD) strain of Venezuelan equine encephalitis (VEE) virus and its live attenuated vaccine derivative, TC-83 virus, have different neurovirulence characteristics. A full-length cDNA clone of the TC-83 virus genome was constructed behind the bacteriophage T7 promoter in the polylinker of plasmid pUC18. To identify the genomic determinants of TC-83 virus attenuation, TRD virus-specific sequences were inserted into the TC-83 virus clone by in vitro mutagenesis or recombination. Antigenic analysis of recombinant viruses with VEE E2- and E1-specific monoclonal antibodies gave predicted antigenic reactivities. Mouse challenge experiments indicated that genetic markers responsible for the attenuated phenotype of TC-83 virus are composed of genome nucleotide position 3 in the 5'-noncoding region and the E2 envelope glycoprotein. TC-83 virus amino acid position E2-120 appeared to be the major structural determinant of attenuation. Insertion of the TRD virus-specific 5'-noncoding region, by itself, into the TC-83 virus full-length clone did not alter the attenuated phenotype of the virus. However, the TRD virus-specific 5'-noncoding region enhanced the virulence potential of downstream TRD virus amino acid sequences.

Venezuelan equine encephalitis (VEE) virus is a mosquito-borne alphavirus (family *Togaviridae*) that contains a single-stranded, positive-sense RNA genome of approximately 11.45 kb. The genome is capped at the 5' end, polyadenylated at the 3' end, and enclosed within an icosahedral nucleocapsid surrounded by a lipid bilayer containing two integral envelope glycoproteins, E1 and E2. The genome organization is 5'-noncoding region (5'-NC)-nonstructural protein 1 (nsP1)-nsP2-nsP3-nsP4-26S junction region-capsid-E3-E2-6K-E1-3'-NC (52). VEE E2 glycoprotein elicits neutralization and hemagglutination inhibition antibodies (13), and E2-specific monoclonal antibodies (MAbs) neutralize the virus to a high titer (45, 47, 48).

The live attenuated TC-83 vaccine strain of VEE virus was derived by serial passage of the equine-virulent, epizootic Trinidad donkey (TRD) strain in fetal guinea pig heart cells (3). After 45 passages, the TRD virus became attenuated for adult mice by both peripheral and intracranial (i.c.) routes of inoculation. Because viruses at the 50th and 80th passage levels caused clinical reactions in 85% of vaccinated humans, a substrain was derived by plaque purifying the 78th passage virus in chicken embryo fibroblasts. Nine of 12 plaque-purified viruses killed 3 to 23% of challenged mice (37). Further guinea pig heart cell passage of one of the three plaque-derived mouse-avirulent viruses resulted in TC-82 virus, which served as stock for preparation of the TC-83 vaccine (36). The protective immunogenic efficacy of the TC-83 vaccine has been demonstrated in humans (4) and equines (55).

TRD virus and its TC-83 vaccine derivative differ in their neurovirulence. TRD virus causes death of laboratory mice within 8 days postinfection independent of dosage or age (3, 15). The median intraperitoneal (i.p.) lethal dose of TRD

virus in weanling or adult Swiss white mice is less than a single PFU. Peripheral challenge of mice with 10^4 or greater PFU of TC-83 virus results in an immunizing infection without clinical symptoms (3, 15, 29, 35).

The TRD and TC-83 virus genomes differ at 12 nucleotide positions: one at position 3 in the 5'-NC; a TRD-to-TC-83 C-to-A substitution at position 1696 (encoding a TRD-to-TC-83 Ala-to-Asp substitution at amino acid position nsP2-16—this difference is a correction to the published data); seven positions encoding nsP3-260 Ser to Thr, E2-7 Lys to Asn, E2-85 His to Tyr, E2-120 Thr to Arg, E2-192 Val to Asp, E2-296 Thr to Ile, and E1-161 Leu to Ile; a single silent mutation in both E2 and E1 genes; and a single nucleotide deletion in the 3'-NC of TC-83 virus (26, 30, 31).

To identify the genetic determinants responsible for the attenuated phenotype of VEE TC-83 virus, we constructed a full-length infectious cDNA clone of TC-83 virus. The virulence characteristics of VEE viruses derived from this clone and from recombinant TC-83-TRD cDNA clones in mice were determined.

MATERIALS AND METHODS

Wild-type viruses. VEE TRD and TC-83 viruses were plaque purified in Vero cell monolayers (13). Passage histories of stock seed viruses used in this study, including plaque purifications, were guinea pig brain-1/Vero-6/ BHK21-1 and TC-83 vaccine/Vero-3/BHK21-1.

Construction of the full-length TC-83 cDNA clone, pVE/IC-92. The full-length, 11,447-bp cDNA clone of VEE TC-83 virus was constructed with cDNA clones of VEE TRD and TC-83 viruses (26, 30, 31). All of the subunit cDNA clones were sequenced. Intermediate and full-length cDNA clones were constructed in the polylinker site of plasmid pUC18. Splicing strategies removed eight artifactual mutations identified in the subunit clones (26, 30, 31). A single TC-83 clone

* Corresponding author.

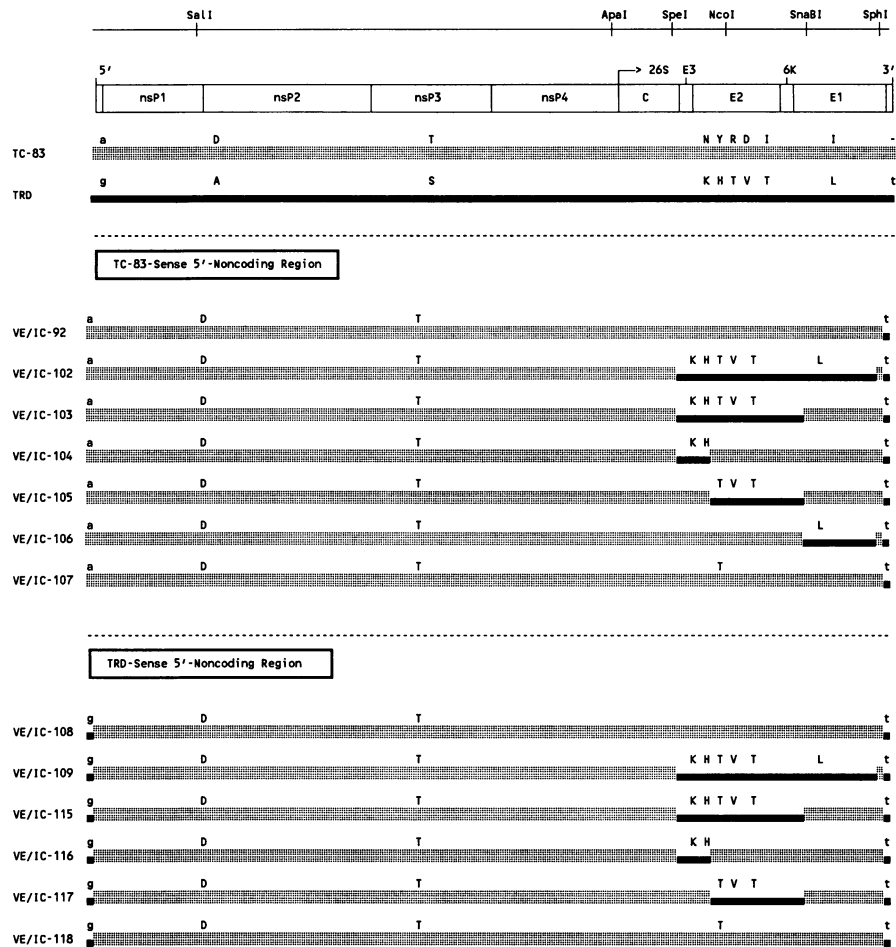


FIG. 1. Summary schematic diagram of pVE/IC-92 and its recombinant full-length cDNA clone derivatives. The genome organization of VEE virus and relevant restriction enzyme splice sites are shown at the top. Virus-specific amino acid (uppercase in translated regions) and nucleotide (lowercase in noncoding regions) residues are indicated above the genome schematics of TC-83, TRD, and recombinant VE/IC viruses. The cDNA regions derived from pVE/IC-92 are indicated by wide, lightly stippled bars. Those regions resulting from insertion of appropriate domains of TRD virus cDNA into the pVE/IC-92 backbone are indicated by narrow, solid bars. All of the full-length cDNA clones have the TRD virus-specific 3'-NC (TRD t versus TC-83 deletion (-) at TRD nucleotide position 11409).

artifact, a C-to-T error at genome position 10356, resulting in an E1-119 Ala-to-Val substitution, remained in the final full-length construct, pVE/IC-92. Clones 5'-pTC46-pTC19-pTRD30-pTC9-pTRD26-pTC5-3' were spliced together at restriction enzyme sites *SalI* (genome position 1620)-*BglII* (2258)-*SstI* (3628)-*SstI* (5550)-*ApaI* (7501)-*SphI* (11377). TRD virus-specific cDNA regions were identical in nucleotide sequence to the cognate regions of the TC-83 virus genome.

The 3' end of the clone, which contained a poly(A-T)₂₅ tail followed by a unique *MluI* site and an *EcoRI* site, was incorporated into the clone by ligation of two annealed complementary synthetic DNA strands containing *SphI* and *EcoRI* single-stranded DNA overhangs. All of the pVE/IC constructs in this study contained the TRD virus 3'-NC. The 5' terminus of the clone was engineered by in vitro mutagenesis (5) of pTC-46 to incorporate the bacteriophage T7 promoter. The terminal sequence of this T7GpTC-46 clone was 5'-TCTAGA/AATTTAATACGACTCACTATA/G/A (*XbaI*/T7 promoter/extraneous G/VEE 5' terminus). Incorporation of an extraneous G residue between the T7 promoter and the VEE 5' end was based on the demonstration that transcription efficiency is greater with a G residue,

rather than an A residue, at the +1 position relative to the promoter (41). All constructs having the TC-83 virus-specific 5'-NC, except for pVE/IC-102G⁻, contained this extraneous G nucleotide. The extraneous residue was deleted in the pVE/IC-102G⁻ construct. Three silent mutations were engineered into T7GpTC-46 at VEE genome nucleotide positions 1613 (A to G), 1616 (C to A), and 1619 (T to C) to facilitate the identification of clone-derived virus.

Recombinant TC-83-TRD full-length clones were constructed by splicing appropriate TRD virus-specific cDNA fragments into the pVE/IC-92 backbone (Fig. 1). A single Arg-to-Thr amino acid substitution at position E2-120 in pVE/IC-107 was made by in vitro mutagenesis (5). To determine the effect of the TRD 5'-NC, the T7GpTC-46 cDNA in M13mp19 was mutagenized (53) (T7-GEN kit; United States Biochemical Corp., Cleveland, Ohio) to incorporate the TRD virus-specific G residue at genome position 3 and remove the extraneous G nucleotide between the promoter and the VEE 5' terminus.

Accuracy of full-length constructs was confirmed by appropriate nucleotide sequencing and restriction enzyme mapping. Cloning, sequencing, and CsCl purification of

recombinant plasmids were performed as described previously (26, 30, 31). Calcium chloride-competent or electroporation-competent (10) *E. coli* XL1-Blue cells (Stratagene Cloning Systems, La Jolla, Calif.) were used in cloning protocols.

Derivation of VE/IC viruses. Infectious, positive-sense, full-length VE/IC genomic mRNA was transcribed from 0.1 to 2.0 μ g of CsCl-purified pVE/IC plasmid with a commercial RNA transcription kit (Stratagene). Plasmid was linearized at the 3' end of the VEE virus-specific cDNA insert by digestion with *Mlu*I, digested with proteinase K (4 mg/ml), extracted with phenol-chloroform-isoamyl alcohol (25:24:1), extracted with ether, and precipitated with ethanol. Transcriptions were performed in reaction volumes of 0.05 ml containing 1,000 U of bacteriophage T7 RNA polymerase (Stratagene) per ml, 0.4 mM (each) ATP, CTP, GTP, and UTP (Stratagene or Pharmacia-LKB Biotechnology, Piscataway, N.J.), 0.25 mM m⁷GpppA cap analog (Pharmacia-LKB), 1600 U of RNase inhibitor (Promega Corporation, Madison, Wis.) per ml, 5 mM dithiothreitol, and 1.0 mg of acetylated bovine serum albumin per ml in 40 mM Tris-HCl (pH 8.0), 8 mM magnesium chloride, 2.0 mM spermidine, and 50 mM sodium chloride. Inclusion of cap analog in the reaction mixture has been shown to increase the transfection efficiency of transcribed Sindbis virus RNA (43). After incubation for 30 min at 37°C, the entire reaction volume was mixed with 0.05 ml of the cationic lipid Lipofectin (GIBCO BRL, Life Technologies, Inc., Gaithersburg, Md.), incubated for 15 min at ambient temperature, and added to a 75-cm² flask of freshly confluent BHK-21 cells containing 3.0 ml of serum-free Eagle's minimal essential medium. After incubation for 8 to 16 h at 37°C, 3.0 ml of the same medium containing 10% (vol/vol) fetal bovine serum (GIBCO BRL) was added. Incubation was continued until 3 to 5 days posttransfection, when cytopathic effects were present in 20% or more of the transfected cells. Medium was collected, made 10% in fetal bovine serum, clarified by centrifugation, and stored in aliquots at -70°C. The resulting VE/IC viruses were tested at this point or after another passage in BHK-21 cells. Virus titers were determined by plaque titration in six-well plates of Vero cell monolayers.

Mouse challenge experiments. Male Swiss ICR mice, 21 to 24 days or 6 weeks old, were challenged i.p. with 5 to 200 PFU of VEE TRD, VEE TC-83, or VE/IC virus. Twenty-one days postinfection, surviving mice were bled from the retroorbital sinus. One or 2 days later, survivors received a secondary i.p. challenge with 40 PFU of TRD virus to determine their protective immune status. i.c. challenges were performed by inoculating methoxyflurane (Metofane; Pitman-Moore, Mundelein, Ill.)-anesthetized 4-week-old mice i.c. with 0.03 ml of diluent containing 200 PFU of virus.

Serology. Serum dilution-plaque reduction neutralization tests were performed in six-well plates of Vero cells (18). Indirect immunofluorescence tests were performed with acetone-fixed virus-infected Vero cells by using fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (SIGMA) and VEE virus-specific MAbs or polyvalent immune sera. MAbs used included 5B4D-6 (epitope E2^a), 1A4A-1 (E2^c), 1A4D-1 (E2^f), 1A3A-9 (E2^g), 1A3B-7 (E2^h), 1A2B-10 (E2ⁱ), 3B2D-5 (E1^a), 3B2A-9 (E1^b), and 3A5B-1 (E1^d) (44, 45, 48). Enzyme-linked immunosorbent assays (ELISAs) were performed by using gradient-purified virus as an antigen (46).

Growth curves in cell culture. Growth curves were performed in 75-cm² tissue culture flasks of confluent BHK-21 cell monolayers. Virus inoculum was adsorbed at a multi-

licity of infection of 0.003 PFU per cell for 1.5 h in a volume of 1 ml. The inoculum was then removed, the cell monolayer was rinsed once with 10 ml of phosphate-buffered saline, and 30 ml of Eagle's minimum essential medium containing 5% (vol/vol) fetal bovine serum (GIBCO Laboratories, Grand Island, N.Y.) was added. Cell cultures were incubated at 37°C. Aliquots (0.2 ml) of culture medium were removed at appropriate intervals and stored at -70°C prior to plaque titration in Vero cell monolayers.

Nucleotide sequence accession number. The nucleotide sequences of the TRD and TC-83 virus genomes, including recent corrections, have been submitted to the GenBank data base (accession numbers L01442 and L01443, respectively).

RESULTS

Plaque sizes of VE/IC viruses. Mean diameters of virus plaques in Vero cells were determined (Table 1). The 2.8-mm plaque size of VE/IC-92 virus was essentially identical to that of TC-83 virus. Substituting TRD virus-specific moieties containing the E2-TV^T triad or E2-120 Thr into the pVE/IC-92 genotype resulted in recombinant viruses that produced significantly ($P = 0.05$ level, Student's independent t test) larger plaques of 3.4 to 4.3 mm. The TRD virus E2-KH diad alone resulted in significantly smaller 2.1-mm plaques in VE/IC-104 virus.

The presence of the TRD virus 5'-NC had little effect on plaque size in comparison to VE/IC viruses containing the TC-83 virus 5'-NC. However, the mean diameters of VE/IC-115 and -118 virus plaques were significantly larger than those of VE/IC-103 and -107 viruses, respectively. The 5.7-mm mean plaque diameter of TRD virus was significantly larger than that of any recombinant VE/IC virus.

Epitope maps of VE/IC viruses. A panel of VEE virus E2- or E1-specific murine MAbs was used to evaluate expression of VEE epitopes in virus-infected Vero cells. E1-specific MAbs defining epitopes E1^a, E1^b, and E1^c gave positive reactions when tested by indirect immunofluorescence with cells infected with TC-83, TRD, and VE/IC-92, -102, -103, -104, -105, -107, -108, and -109 viruses (data not shown). Endpoint titrations of E2-specific MAbs were performed with these viruses, as well as VE/IC-108, -109, -115, -116, -117, and -118 viruses (Table 2). The MAbs defining VEE epitopes E2^c (Table 2), E2^f, E2^g, and E2^h (data not shown) were reactive to a high titer with all of the viruses. The MAb defining E2^c neutralizes VEE virus to a high titer (45, 48). A control MAb, 6B6C-1, specific for the envelope glycoprotein of St. Louis encephalitis virus, showed no reactivity (data not shown).

The MAb 1A2B-10, which defines the E2ⁱ epitope present on all tested wild-type VEE viruses, except for TC-83 and VEE subtype VI virus (44), gave reciprocal titers of 25,600 to 51,200 with cells infected with viruses that contained the TRD virus E2-KH diad (VE/IC-102, -103, -104, -109, -115, and -116 and TRD). The anti-E2ⁱ MAb failed to react with cells infected with viruses containing the TC-83 virus E2-NY diad (VE/IC-92, -105, -107, -108, -117, and -118 and TC-83). Conversely, the TC-83 virus-specific MAb 5B4D-6 defining the E2^a epitope reacted to a reciprocal titer of 3,200 to 12,800 only with cells infected with viruses containing the TC-83 virus E2-NY diad and with VE/IC-109 virus. Multiple tests of anti-E2^a MAb antibody with cells infected with VE/IC-109 virus or VE/IC-109B virus, which was derived from pVE/IC-109 in a second transcription-transfection protocol, consistently gave reactivities equivalent to or only slightly lower

TABLE 1. Mean plaque diameters of viruses 6 days postinfection in Vero cells

Virus	Mean (SE) diam ^a	Genotype ^b										
		a	D	S	N	Y	R	D	I	A	I	*
TC-83	2.7 (0.4)	a	D	S	N	Y	R	D	I	A	I	*
VE/IC-92	2.8 (0.3)	V	.	t
VE/IC-102	4.1 (0.5)	.	.	.	K	H	T	V	T	.	L	t
VE/IC-102G ⁻	4.3 (0.6)	.	.	.	K	H	T	V	T	.	L	t
VE/IC-103	3.7 (0.9)	.	.	.	K	H	T	V	T	V	.	t
VE/IC-104	2.1 (0.4)	.	.	.	K	H	.	.	.	V	.	t
VE/IC-105	3.6 (0.5)	T	V	T	V	.	t
VE/IC-107	3.4 (0.5)	T	.	.	V	.	t
VE/IC-108	3.0 (0.4)	g	V	.	t
VE/IC-109	4.6 (0.6)	g	.	.	K	H	T	V	T	.	L	t
VE/IC-115	5.1 (0.6)	g	.	.	K	H	T	V	T	V	.	t
VE/IC-116	1.8 (0.3)	g	.	.	K	H	.	.	.	V	.	t
VE/IC-117	4.4 (0.5)	g	T	V	T	V	.	t
VE/IC-118	3.8 (0.4)	g	T	.	.	V	.	t
TRD	5.7 (0.6)	g	A	T	K	H	T	V	T	.	L	t

^a Mean diameter in millimeters of 12 plaques.

^b The TC-83 virus genotype is a-D-S-NYRDI-I* (5'-NC-nsP2-nsP3-E2-E1-3'-NC deletion). Δ, correct residue at TC-83 and TRD amino acid position E1-119 (26, 30); V, cDNA artifact incorporated at E1-119 in plasmid pVE/IC-92; dot (·), identity with TC-83 virus.

than with TC-83 virus. This result was unexpected, because these viruses contained the E2 amino-terminal sequences of TRD virus and were reactive with anti-E2¹ MAb. To investigate this anomaly further, the MAb reactivities of several gradient-purified viruses were tested by ELISA. In the ELISA, purified TRD and VE/IC-102G⁻, -102, -109, and -109B viruses reacted only with E2²-specific MAb. These viruses failed to react with anti-E2^a MAb. Both TC-83 and VE/IC-92 viruses reacted to a reciprocal titer of 32,000 with anti-E2^a MAb but failed to react with anti-E2² MAb (data not shown). The reason for the anomalous reactivity of VE/IC-109 virus-infected cells by immunofluorescence was undetermined.

Mouse survival following i.p. VE/IC virus challenge. To investigate virulence characteristics of the recombinant VE/IC viruses, 3- and 6-week-old mice were challenged i.p.

with 200 PFU of TRD, TC-83, or VE/IC virus (Table 3). VEE TC-83 and VE/IC-92, -104, and -106 viruses were avirulent. VE/IC-102, -103, and -105 viruses, which contained the TC-83 virus 5'-NC and TRD virus E2-TVT triad, caused lethal infections with average survival times of 9 to 10 days in 50 to 100% of challenged 3-week-old mice. Older, 6-week-old mice were more resistant. The extraneous G nucleotide between the T7 promoter and VEE 5' end genomic nucleotide in plasmid constructs containing the TC-83 virus 5'-NC had no apparent effect on the virus derivatives, as shown by the phenotypic identity between VE/IC-102 and -102G⁻ viruses in terms of plaque size (Table 1) and virulence for mice (Table 3). VE/IC-107 virus, which contained the single TRD virus E2-120 Thr substitution in the VE/IC-92 virus background, showed low-level virulence in 3-week-old mice and was avirulent for 6-week-old mice.

TABLE 2. Analysis of antigenic structures of viruses^a

Virus	Titer of antibody-defining epitope ^b			Genotype ^c										
	E2 ^a	E2 ¹	E2 ^c	a	D	S	N	Y	R	D	I	A	I	*
TC-83	64	—	512	a	D	S	N	Y	R	D	I	A	I	*
VE/IC-92	64	—	512	V	.	t
VE/IC-102	2	256	512	.	.	.	K	H	T	V	T	.	L	t
VE/IC-103	4	256	512	.	.	.	K	H	T	V	T	V	.	t
VE/IC-104	2	256	512	.	.	.	K	H	.	.	.	V	.	t
VE/IC-105	64	—	1,024	T	V	T	V	.	t
VE/IC-107	32	—	2,048	T	.	.	V	.	t
VE/IC-108	32	—	1,024	g	V	.	t
VE/IC-109	64	512	512	g	.	.	K	H	T	V	T	.	L	t
VE/IC-115	4	512	1,024	g	.	.	K	H	T	V	T	V	.	t
VE/IC-116	2	512	1,024	g	.	.	K	H	.	.	.	V	.	t
VE/IC-117	128	—	256	g	T	V	T	V	.	t
VE/IC-118	128	—	512	g	T	.	.	V	.	t
TRD	2	256	256	g	A	T	K	H	T	V	T	.	L	t

^a Analysis is by direct immunofluorescence of acetone-fixed, virus-infected Vero cells by using VEE virus E2-specific MAbs.

^b Reciprocal titer/100 by using twofold dilution series of MAbs standardized to 1 mg/ml. —, ≤1.

^c See footnote b to Table 1.

TABLE 3. Survival of white male Swiss ICR mice after i.p. challenge with 200 PFU of virus

Virus	3-wk-old mice		6-wk-old mice		Virus genotype ^c														
	% Mortality ^a	Avg (SE) survival ^b (days)	% Mortality ^a	Avg (SE) survival ^b (days)															
Diluent	0.0	—																	
TC-83	0.0	—	0.0	—	a	D	S	N	Y	R	D	I	A	I	*				
VE/IC-92	0.0	—	0.0	—	V	.	t				
VE/IC-102	87.5	9.4 (0.6)	25.0	12.0 (0.0)	.	.	.	K	H	T	V	T	.	L	t				
VE/IC-102G ⁻	87.5	9.0 (0.0)	50.0	11.5 (0.5)	.	.	.	K	H	T	V	T	.	L	t				
VE/IC-103	100.0	9.4 (0.3)	12.5	16.0	.	.	.	K	H	T	V	T	V	.	t				
VE/IC-104	0.0	—	0.0	—	.	.	.	K	H	.	.	.	V	.	t				
VE/IC-105	62.5	9.8 (0.4)	37.5	13.3 (1.9)	T	V	T	V	.	t				
VE/IC-107	37.5	10.7 (0.3)	0.0	—	T	.	.	V	.	t				
VE/IC-106	0.0	—	0.0	—	L	t				
VE/IC-108	0.0	—	0.0	—	g	V	.	t				
VE/IC-109	100.0	6.0 (0.0)	100.0	6.0 (0.0)	g	.	.	K	H	T	V	T	.	L	t				
VE/IC-115	100.0	6.1 (0.2)	100.0	6.0 (0.0)	g	.	.	K	H	T	V	T	V	.	t				
VE/IC-116	0.0	—	0.0	—	g	.	.	K	H	.	.	.	V	.	t				
VE/IC-117	100.0	6.8 (0.1)	100.0	6.4 (0.2)	g	T	V	T	V	.	t				
VE/IC-117B	100.0	6.0 (0.0)	100.0	6.0 (0.0)	g	T	V	T	V	.	t				
VE/IC-118	100.0	6.6 (0.2)	100.0	6.5 (0.2)	g	T	.	.	V	.	t				
VE/IC-118B	100.0	6.6 (0.2)	100.0	7.8 (0.7)	g	T	.	.	V	.	t				
TRD	100.0	5.0 (0.0)	100.0	6.1 (0.3)	g	A	T	K	H	T	V	T	.	L	t				

^a Percent mortality of eight mice per group.
^b Average survival time + standard error to total paralysis or death. —, not applicable (no mouse deaths).
^c See footnote b to Table 1.

Substitution of the TRD virus 5'-NC into the genome of VE/IC-92 and -104 viruses, which contained the TRD virus E2-KH diad, did not affect their attenuated phenotype; VE/IC-108 and -116 viruses were avirulent (Table 3). However, the combination of TRD virus 5'-NC and E2-TVT triad in VE/IC-109, -115, and -117 viruses resulted in increased virulence, as evidenced by consistent 100% lethality and shorter average survival times of 6 to 7 days in 3-week-old mice and 6 to 8 days in 6-week-old mice (Table 3). The presence of the TRD virus 5'-NC and E2-120 Thr moieties in VE/IC-118 virus reconstituted most of the TRD virus virulence. Average survival times were shorter in 3-week-old mice challenged with TRD virus than in those challenged with any of the VE/IC viruses. However, similar survival times were observed for 6-week-old mice challenge with TRD and VE/IC-109, -115, -117, and -118 viruses.

Percent mortality and average survival times in 3-week-old mice challenged with 5 to 13 PFU of VEE TRD or VE/IC-102 or -109 virus were essentially identical to those following challenge with 200 PFU of the corresponding virus (data not shown). Three-week-old mice challenged i.p. with 5 or 14 PFU of TRD-trans virus, derived by transfection of BHK-21 cells with wild-type TRD virus RNA, had average survival times of 6.2 (±0.3) or 5.4 (±0.5) days, respectively (data not shown). Mung bean nuclease treatment of *Mlu*I-linearized pVE/IC-102 or -109 plasmid DNA prior to RNA transcription and transfection had no effect on the virulence phenotype of VE/IC-102 or -109 virus (data not shown). The virulence of plaque-purified VE/IC-102 or -109 virus for 3-week-old mice by the i.p. route was essentially identical to that of the uncloned parent virus. However, TRD-trans cloned virus was attenuated relative to wild-type TRD parent virus. One mouse survived challenge with the cloned

derivative of TRD-trans virus, while the remaining seven mice in the group survived an average of 7.9 (±0.1) days (data not shown).

All mice that survived primary i.p. virus challenge (Table 3) received a secondary i.p. challenge with 40 PFU of TRD virus 23 or 33 days post-primary challenge. Except for three of the eight VE/IC-116 survivors, all mice that survived primary virus challenge also survived secondary TRD virus challenge.

Mouse survival after i.c. VE/IC challenge. Susceptibility of 4-week-old mice to i.c. challenge with 200 PFU of VE/IC virus is shown in Table 4. Three of the eight mice challenged with TC-83 or VE/IC-92 virus died 9 to 10 days after i.c. challenge. Mice challenged with VE/IC-102, -105, or -107 virus demonstrated 87.5 to 100% mortality, with average survival times of 9 days in those that died. VE/IC-104, -108, and -116 viruses, which were avirulent by peripheral inoculation (Table 3), were fatal for 62.5, 50, and 25%, respectively, of the mice challenged by the i.c. route. Interestingly, VE/IC-116 virus, which contained the TRD virus 5'-NC and E2-KH diad, killed fewer mice by the i.c. route than did VE/IC-104 virus, which contained the TC-83 virus 5'-NC. VE/IC-109, -115, -117, and -118 viruses were as lethal or nearly as lethal as TRD virus by the i.c. route. All mice that survived i.c. challenge with VE/IC virus possessed reciprocal neutralization antibody titers of 40 or greater, indicating that virus replication did occur in these animals (data not shown).

Immune responses of mice challenged with VE/IC virus. In ELISAs with purified TC-83 virus as an antigen, pooled sera from 3- or 6-week-old mice surviving challenge with TC-83 or VE/IC-92, -102, -103, -104, -105, -107, or -109 virus gave reciprocal endpoint titers of 640 to 10,240. Titers increased

TABLE 4. Survival of 4-week-old white male Swiss ICR mice following i.c. challenge with 200 PFU of virus^a

Virus	% Mortality	Avg (SE) survival (days)	Virus genotype					
Diluent	0.0	—						
TC-83	37.5	9.7 (0.9)	a	D	S	N Y R D I	A I	*
VE/IC-92	37.5	10.3 (0.5)	V .	t
VE/IC-102	100.0	8.9 (0.3)	.	.	.	K H T V T	. L	t
VE/IC-104	62.5	10.0 (0.0)	.	.	.	K H . . .	V .	t
VE/IC-105	100.0	9.0 (0.5) T V T	V .	t
VE/IC-107	87.5	8.9 (0.3) T . .	V .	t
VE/IC-108	50.0	9.3 (0.4)	g	V .	t
VE/IC-109	100.0	5.0 (0.0)	g	.	.	K H T V T	. L	t
VE/IC-115	100.0	5.4 (0.5)	g	.	.	K H T V T	V .	t
VE/IC-116	25.0	8.0 (1.0)	g	.	.	K H . . .	V .	t
VE/IC-117	100.0	5.9 (0.9)	g T V T	V .	t
VE/IC-118	100.0	5.8 (0.4)	g T . .	V .	t
TRD	100.0	5.0 (0.0)	g	A	T	K H T V T	. L	t

^a See footnotes b to Table 1 and a and b to Table 3.

less than fourfold, remained stable, or decreased 2 weeks after secondary TRD virus challenge, except for one group challenged with VE/IC-102 virus, whose serum showed an increase in reciprocal titer from 2,560 to 10,240 (data not shown). By cross-neutralization testing, reciprocal antibody endpoints resulting in 70% or greater reduction of 28 to 89 PFU of these viruses were at least 320 for all pooled sera obtained after primary challenge with these viruses (data not shown).

Growth curves of VE/IC viruses in cell culture. The growth characteristics of VEE TRD, TC-83, and VE/IC-92, -109, -108, -102G⁻, -107, and -118 viruses in BHK-21 cells were

compared (Fig. 2). The growth curves of these viruses were similar in shape and magnitude. Cytopathic effect at 76 h postinfection was scored as +2, +1, +1, +1, and +1 for cells infected with TC-83, VE/IC-92, VE/IC-108, VE/IC-102G⁻, and VE/IC-107 virus, respectively, and as +2, +4, and +4 for TRD, VE/IC-109, and VE/IC-118 viruses, respectively. All viruses produced +4 or greater cytopathic effect by 108 h postinfection.

Attenuating effect of a mutation at E2-209. The original pTRD-1 26S cDNA clone of TRD virus contained a cDNA "error," resulting in the substitution of Lys for the correct Glu residue at amino acid position E2-209 (30) and the loss of

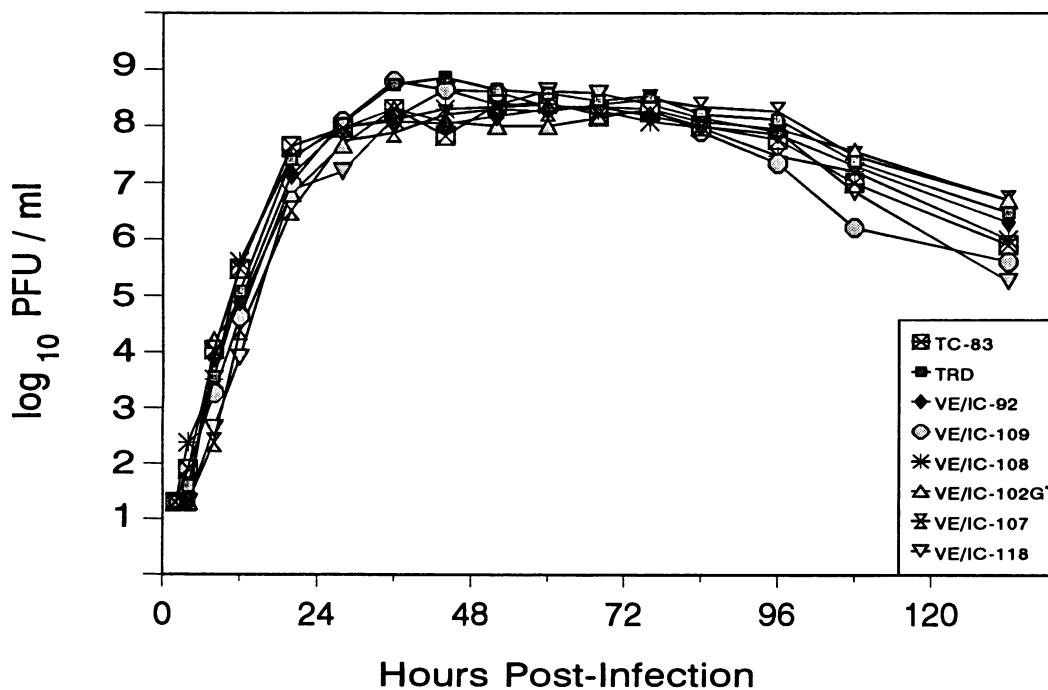


FIG. 2. Growth curves of VEE viruses in BHK-21 cells. Multiplicity of infection was 0.003 PFU per cell.

the E2^h epitope (28). This clone was corrected to incorporate Glu at amino acid position E2-209 prior to constructing the recombinant TC-83/TRD viruses shown in Tables 1 to 4. To determine the effect of this mutation, the *SpeI* (8895)-*SphI* (11377) fragment (Fig. 1) of the original pTRD-1 clone was substituted for the cognate region of pVE/IC-92 and -109, which resulted in VE/IC-101 and -114 viruses, respectively. Mean plaque diameters of VE/IC-101 and VE/IC-114 viruses were 4.0 mm (± 0.7 mm) and 4.5 mm (± 0.6), which were similar to the diameters of VE/IC-102 and -109 virus plaques, respectively (Table 1). MAb 1A3B-7, which defines the E2^h epitope, did not react by immunofluorescence with Vero cells infected with either VE/IC-101 or -114 virus (data not shown). The single E2-209 Glu-to-Lys substitution completely attenuated the VE/IC-101 virus for 3-week-old mice challenged i.p. Addition of the TRD virus 5'-NC in VE/IC-114 virus caused partial reversion of this attenuating mutation, but the highly virulent phenotype of VE/IC-109 virus was not reestablished. i.p. challenge with VE/IC-114 virus was lethal for 75% of 3-week-old mice in 8.5 (± 1.0) days and 12.5% of 6-week-old mice in 12.0 days. By the i.c. route, the virulence phenotypes of VE/IC-101 and -114 viruses were essentially equivalent to those of VE/IC-102 and -109 viruses, respectively.

DISCUSSION

To determine the genetic loci defining TC-83 virus attenuation, we engineered an infectious cDNA clone of this virus. Phenotypic identity between VE/IC-92 and TC-83 viruses with respect to plaque morphology, antigenic structure, avirulence by i.p. inoculation of mice, level of virulence by the i.c. route, and growth in BHK-21 cells indicated that the VEE virus-specific cDNA in pVE/IC-92 was representative of the genome of TC-83 virus. Recombinant VE/IC viruses expressed the appropriate E1 and E2 epitopes. MAb reactivities with the chimeric viruses suggested that the E2^a and E2ⁱ epitopes reside within the amino-terminal 85 amino acids of the E2 protein. The E2ⁱ epitope has been previously identified as being defined by TRD E2-7 Lys (44).

The virulent phenotype of TRD virus was reconstituted almost entirely in TC-83-TRD chimeras by two TRD virus-specific genetic loci: the G residue at genome nucleotide position 3 in the 5'-NC and amino acid E2-120 Thr. Although partial reversion to virulent phenotype occurred with the TRD virus E2 alone, the TRD virus 5'-NC by itself did not change the attenuated phenotype of VE/IC-92 virus. The TRD virus E2-192 Val and/or E2-296 Thr residue in the E2-TVT triad appeared to contribute to the virulent phenotype. Amino acid position E2-192 is located within the neutralization domain between residues E2-182 and E2-207 defined by MAb escape variants of Sindbis and VEE viruses (8, 25, 49-51). Attenuation by only one or two mutations may explain the rapid reversion to virulence of TC-83 virus in mice (3) and hamsters (23).

Since the average survival time of 3-week-old mice challenged i.p. with TRD virus was generally shorter and plaques in Vero cells inoculated with TRD virus were larger than with any recombinant VE/IC virus, virulence may be further modulated by other genetic loci or uncharacterized variants in the TRD virus population. Adding the TRD virus nsP3-260 Thr to the VE/IC-109 genotype resulted in VE/IC-113 virus, which was equivalent to VE/IC-109 virus in virulence for mice (data not shown). Therefore, the most likely locus for further modulation of virulence is amino acid nsP2-16. Since mice challenged i.p. with TRD-trans virus also had longer

survival times, the extended survival time relative to that of wild-type TRD virus may reflect the nature of virus populations derived by transfection of BHK-21 cells with genomic RNA.

Synergism among multiple genetic loci in virus virulence is well established. Attenuation in poliovirus has been shown to involve several genetic loci, including the 5'-NC (42). Poliovirus type 3 vaccine can revert to virulence via a single point mutation in the 5'-NC (11). Virulence markers of Theiler murine encephalitis virus were mapped to a 5' end genome segment, which was avirulent by itself, and a capsid segment (14). Passage of Ross River virus in mice resulted in selection of a more virulent variant that had several amino acid substitutions in a neutralization domain (40). Different combinations of substitutions in the E1 and E2 proteins of Sindbis virus have also resulted in the generation of viruses having variable virulence (34). Using an independently derived infectious clone of VEE TRD virus, Davis et al. (9) demonstrated that single point mutations at E2 amino acid position 3, 4, 76, 120, or 209 abrogated virulence for adult mice and that the attenuating effect of combined mutations was additive.

VEE virus virulence correlates well with high and prolonged viremia in equines (27, 54) and laboratory rodents (20, 24, 33). In mice, infection with virulent VEE virus causes marked lymphocytic depletion and necrosis in the spleen, lymph nodes, and bone marrow. Mice infected with virulent virus develop encephalitis that involves the entire brain, spinal cord, and meninges, with severe perivascular cuffing, gliosis, and necrosis of neurons, especially the large motor neurons in the spinal cord (15-17). However, mice challenged peripherally with the attenuated TC-83 virus develop lower virus titers in the blood and brain and are spared the histopathological lesions that occur in the spleen, lymph nodes, and brain tissue of mice infected with virulent VEE virus (33).

The fatal outcome of virulent VEE virus infection in laboratory rodents appears to be related to efficient virus replication and extensive histopathology in various tissues. The synergism between the 5'-NC and E2 envelope glycoprotein suggests that a dual mechanism is involved in the attenuation of TC-83 virus. The E2 contribution may be a surface epitope of the virus that is involved in cell attachment and entry with concomitant effects on virus clearance rate (20, 21, 22, 24, 32) and host cell tropism. An E2-120 Thr-to-Lys and E2-114 Ser-to-Arg mutation in rapid penetration variants of VEE and Sindbis viruses, respectively, coincided with virus attenuation (7, 9). Avirulent strains of Semliki Forest virus lose the ability to damage neurons or to enter the brain tissue of infected mice (2).

The 5'-NC of VEE virus may affect replication or translation of viral genomic mRNA in the infected cell. Mecham and Trent (38, 39) demonstrated that TRD and TC-83 viruses had similarly shaped one-step growth curves in Vero cells, but TRD virus produced more virus-specific RNA and inhibited host cell protein synthesis to a greater extent during the first 12 h of the replication cycle. The avirulent A7 strain of Semliki Forest virus synthesizes less viral RNA than do virulent strains in cultured mouse neuroblastoma cells (1), and avirulent strains of Semliki Forest virus fail to produce cytopathologic changes in neuroblastoma cells (2). Replication rate alone may be a primary determinant of neurovirulence. Virulent and avirulent Sindbis viruses infect both neurons and ependymal cells in the central nervous system of mice, but virulent strains infect more cells and produce greater cytopathic effect (19). Fazakerley et al. (12)

reported that attenuation of a variant of mouse hepatitis virus was due to slower spread and decreased neuron destruction rather than to different cell tropism in the central nervous system. Similar results with an attenuated strain of rabies virus were reported (6). The attenuated phenotype of VEE TC-83 virus *in vivo* appears to be the result of mutations in the E2 gene, which may limit infection of specific cell types and increase virus clearance, and in the 5'-NC of the genome, which may limit the extent of virus replication and virus inhibition of cellular functions in infected cells, thereby permitting more time for immunologic intervention in the infected host.

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