COMPARATIVE NEUROVIRULENCE OF ATTENUATED AND NON-ATTENUATED STRAINS OF VENEZUELAN EQUINE ENCEPHALITIS VIRUS IN MICE

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Abstract. A candidate live-attenuated virus vaccine for protection against Venezuelan equine encephalitis (VEE) (designated V3526) was tested in mice to measure the magnitude, duration, and kinetics of virus replication in the blood and the central nervous system and its phenotypic stability after multiple passages in mice and cell culture. All results were compared to parallel experiments with parental virus and the existing VEE virus vaccine, TC-83. Maximum virus titers in the brains of V3526-inoculated mice were between 10^- and 100-fold less than those observed in brains of mice inoculated intracranially (ic) with either the parental virus or TC-83. Neither V3526 nor TC-83 was lethal in BALB/c mice inoculated ic. However, mice inoculated with TC-83 developed acute symptoms lasting at least 14 days. In contrast, ic inoculation of TC-83 was uniformly lethal for C3H/HeN mice. V3526 was avirulent in both BALB/c and C3H/HeN mice after ic inoculation. The virulence characteristics of V3526 remained unchanged after five serial ic passages in mouse brains or after five cell culture passages. Finally, pathologic changes induced after ic inoculation of V3526 were consistently less severe and of shorter duration than those observed in TC-83-inoculated mice. Based on these results, V3526 is stable and appears to be significantly less neurovirulent in mice than TC-83.

INTRODUCTION

The Venezuelan equine encephalitis (VEE) virus complex is composed of serologically related mosquito-borne viruses in the genus Alphavirus, family Togaviridae. Viruses from the VEE-complex have been responsible for extensive epidemics in North, Central, and South America and are highly pathogenic for both humans and members of the family Equidae.1 VEE complex viruses are grouped, based on their epidemiology and ecology, into epizootic and enzootic varieties. All of the epizootic strains are exotic to the United States2 and, until 1995, had not surfaced during natural epidemics since 1973. Between April and October of 1995, an outbreak of fatal encephalitis caused by epizootic VEE virus occurred in Venezuela and Colombia. The outbreak involved over 75,000 human cases and resulted in more than 20 deaths.3,4

Like the other alphaviruses, VEE virus is an enveloped virus, consisting of three structural proteins: capsid (C), which encapsidates the viral genome, and two envelope glycoproteins (E1 and E2). The glycoproteins project from the virus envelope as spikes, each formed from three copies of an E1/E2 heterodimer. These structural proteins are expressed as one large polyprotein from a 26S, subgenomic RNA and the order of translation is C, PE2, 6K, and E1. PE2 is a direct precursor of E2 and includes at its N-terminus 59 amino acid residues (E3) which are removed late in maturation by a Golgi-resident, furin-like enzyme.5

The U.S. Army developed a live-attenuated vaccine (TC-83) for use in humans in the early 1960s.6 Subsequent to its development, the vaccine was also approved for, and distributed for use in horses. The vaccine was developed by classical cell-culture passage techniques, and is currently available for use in humans only under an investigational new drug (IND) protocol. Because the vaccine is not licensed, its use is limited to at-risk laboratory workers, field biologists, and certain other groups of individuals deemed to be at risk for infection.

While TC-83 is normally quite effective, it also has several important disadvantages that ultimately prevent it from being licensed for general use.7 First, approximately 20% of vaccinees will develop some degree of illness after vaccination.8 Five percent of these individuals will develop a relatively-severe febrile disease that resembles naturally acquired VEE. In addition to its high reactogenicity rate, TC-83 also has a high vaccine failure rate, as another 20% of vaccine recipients fail to mount any detectable immune response.6 Rodent-virulent virus can be isolated from throat swabs of some vaccine recipients, indicating that reversion, or at least selection of virulent virus subpopulations occurs after vaccination (Jahrling PB, unpublished data). The vaccine also causes fetal infection and wastage in rodents.9 Additionally, viremias in horses, mules, and donkeys may be sufficient to infect mosquitoes that could potentially transmit virus to other susceptible hosts.10 Finally, there is some evidence from accidental laboratory exposures that the vaccine does not adequately protect against infection from heterologous VEE virus subtypes (Jahrling PB, unpublished data).11

A new-generation, live-attenuated VEE vaccine, currently under development, is being prepared to overcome the problems and limitations associated with TC-83. The new vaccine candidate, V3526 was derived from a full-length cDNA clone of the Trinidad donkey strain (TrD) of VEE virus (V3000) modified to contain two independently-attenuating mutations at defined loci.12 These mutations include a deletion of the four amino acid furin recognition site (RKKR) between structural proteins E3 and E2 and a Phe to Ser change at E1 amino acid residue 253. In vitro transcription of V3526 cDNA results in RNA that is fully infectious for cultured cells and produces an attenuated virus possessing PE2 in the mature virion. Rodent and primate testing of this vaccine indicates that it is safe and effective at protecting animals from a wild-type virus challenge by both parenteral and aerosol routes (Pratt WD, unpublished data).13

This paper presents a comparative analysis of the relative neurovirulence of TC-83, V3526, and wild-type TrD virus.
and discusses the advantages of the new vaccine candidate over the existing product.

**MATERIALS AND METHODS**

**Cell lines and viruses.** Cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD). All cell-culture media and supplements were purchased from Gibco-BRL Life Technologies (Gaithersburg, MD), unless otherwise specified. BHK-21 (ATCC CRL 10), VERO 76 (ATCC CRL 1587), and MRC-5 (ATCC CCL 171) cells were maintained at 36°C in minimal essential medium (MEM) with Hanks’ or Earle’s salts, 5% fetal bovine serum (FBS), 2 mM L-glutamine, 200 units/ml penicillin, and 200 μg of streptomycin per ml.

The V3000 and V3526 strains of VEE virus were derived from a full-length cDNA clone of the TrD strain as described.12,14 TC-83 was acquired from the human-use vaccine prepared by the Salk Institute (Swiftwater, PA) and used either directly from the vaccine vial or as an amplified stock passed once in BHK-21 cells. Wild-type TrD virus used in these studies had been passed once in guinea pigs, 14 times in chicken embryo fibroblasts, once in VERO 76 cells, and once in BHK-21 cells. Virus stocks were prepared on monolayers of BHK-21 cells as described.15

**Plaque assays.** Virus in serum samples and brain homogenates was assayed on monolayers of VERO 76 cells. For these assays, 10-fold serial dilutions of each sample were made in Hank’s balanced salt solution (HBSS) containing 2% FBS. One hundred μl of each dilution was then inoculated in duplicate onto monolayers of VERO-76 cells in 6-well culture plates. Inoculated cultures were incubated at 37°C for 1 hr then overlaid with Basal Medium with Earle’s salts (GIBCO-BRL, Gaithersburg, MD) containing non-essential amino acids (GIBCO-BRL, Gaithersburg, MD), 0.6% (w/v) agarose, 10% FBS, 10 mM HEPES, penicillin (100 U/ml), streptomycin (100 μg/ml), gentamicin (50 μg/ml), and amphotericin B (0.25 μg/ml). After 24 hr at 37°C, the cultures were overlaid with the same medium described above containing 0.017% (w/v) neutral red. Plaques were counted after an additional 24 hr at 37°C

**Animal manipulations.** For all studies, 6- to 8-week-old BALB/c or C3H/HeN mice (National Cancer Institute, Frederick, MD) were housed in cages equipped with microisolators. The mice were provided food and water ad libitum.

For studies involving intracranial (ic) inoculation, mice were anesthetized with an intraperitoneal (ip) injection of a solution containing 7.1 mg/ml of sodium pentobarbital and 9.2% ethanol in sterile saline. The anesthesia was delivered at a rate of 10 μl per gram of body weight. The top of the head was disinfected and a small (1–2 mm) incision was made in the skin to the right of the center line proximal to the ears. Using a small, sterilized drill bit, a hole was bored through the skull and 0.025 ml of inoculum was injected into the right hemisphere of the cerebral cortex with a sterile tuberculin syringe. After inoculation, the incision was closed with Vetbond® (3M, St. Paul, MN). Mice were kept warm in a 37°C incubator until they were fully recovered from anesthesia.

For viremia studies, three groups of 50 mice each were inoculated subcutaneously (sc) with 10⁶ PFU of V3526, TC-83, or TrD. At each time point (0, 5, 24, 29, 48, 53, 72, 77, 96, and 101 hr postinoculation [pi]), 10 mice inoculated with each virus were anesthetized and bled from the retroorbital sinus. To avoid unnecessary trauma, each group was bled no more than twice throughout the experimental period. After 30 to 60 min, blood clots were separated from serum by centrifugation and the sera were frozen at −70°C until they were tested by plaque assay.

To determine the median lethal dose (LD₅₀) of virus, groups of 10 mice were inoculated by the ic (ICLD₅₀) or ip (IPLD₅₀) routes with 10-fold serial dilutions of V3526, TC-83, or TrD. Mice were monitored for 21 days for signs of illness or death. LD₅₀ₙₐₜ were calculated by the Reed-Muench method.14 Comparisons of the mean number of days until death were made by using one-way analysis of variance.

To determine the replication kinetics of VEE virus after ic inoculation, groups of 36 mice were inoculated ic with 1 × 10⁶ PFU of TrD, V3000, TC-83, or V3526. Each day pi, three mice from each group were killed and their brains harvested and triturated in cell culture medium to make a 10% (w/v) suspension. Brain suspensions were then frozen at −70°C until they could be tested by plaque assay. Virus loads were determined and expressed as the average virus load (in PFU) per gram of brain.

For virus reversion studies, 10⁶ PFU of TC-83 or V3526 per mouse were inoculated ic into groups of 12 BALB/c mice. On pi day 2, two mice were killed and their brains were harvested. Brains were triturated in MEM without additives to form a 10% (w/v) suspension and the virus load determined as described above. The remaining mice were observed daily for 21 days for signs of illness or death. The next brain passage was initiated by inoculating 10⁶ PFU of virus from the previous brain passage into another group of 12 mice. This process was repeated through five mouse brain passages.

In conducting the research described in this report, the investigators adhered to the “Guide for Care and Use of Laboratory Animals” as promulgated by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

**In vitro reversion studies.** To test for reversion in cultured cells, virus was serially passed through certified MRC-5 cells. TC-83 or V3526 was incubated with monolayers of MRC-5 cells in 75 cm² flasks at a multiplicity of infection (MOI) of 2 to 3. Cells were incubated for 24 hr at 37°C in a 5% CO₂ environment. After a 100% medium replacement, the cells were incubated for an additional 24 hr and the cell culture supernatant was collected. To remove insoluble inhibitors of virus replication (presumably interferon) the virus supernatant was layered over a 20% to 60% (w/w) sucrose gradient and the virus was collected and diluted to the original supernatant volume in cell culture medium. The resulting virus suspension was titrated by plaque assay. This passage process was repeated for a total of five MRC-5 cell culture passages. Virus (10⁵ PFU) from the fifth passage was inoculated ic into 10 BALB/c mice. These mice were observed daily for 21 days for signs of illness or death.
Table 1
Lethality of Venezuelan equine encephalitis viruses in BALB/c mice

<table>
<thead>
<tr>
<th>Virus</th>
<th>IPLD₅₀⁺</th>
<th>MDD⁺</th>
<th>Sick animals</th>
<th>ICLD₅₀⁺</th>
<th>MDD⁺</th>
<th>Sick animals</th>
<th>Sequellae⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>V3000</td>
<td>1.5⁺</td>
<td>10.3⁺</td>
<td>+</td>
<td>1.4⁺</td>
<td>8.3⁺</td>
<td>+</td>
<td>NA</td>
</tr>
<tr>
<td>TRD</td>
<td>0.8⁺</td>
<td>9.3⁺</td>
<td>+</td>
<td>&gt;0.7⁺</td>
<td>6.5⁺</td>
<td>+</td>
<td>NA</td>
</tr>
<tr>
<td>TC-83</td>
<td>&gt;8.6⁺</td>
<td>NA</td>
<td>−</td>
<td>&gt;7.2⁺</td>
<td>NA</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>V3526</td>
<td>&gt;9.2⁺</td>
<td>NA</td>
<td>−</td>
<td>&gt;5.7⁺</td>
<td>NA</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

* Median intraperitoneal lethal dose.  
⁺ Mean day until death.  
* Median intracranial lethal dose.  
* Aggressive behaviour in survivors.  
* Log₁₀ plaque forming units per ml.  
NA = not applicable.

Pathology. Sixty-four BALB/c and 64 C3H/HeN mice were divided equally into two treatment groups per strain. One treatment group from each strain was injected IC with either TC-83 or V3526. The mice were observed daily for clinical signs of disease and two mice from each experimental group were killed at Days 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 13, 15, and 21, after exposure. Animal tissues were immediately fixed by intracardiac infusion with 10% neutral buffered formaldehyde. The mice were then decapitated, the calvarium carefully removed, and brains post-fixed in situ for 7 days in formaldehyde fixative. Each mouse brain was cut transversely into six sections (one section of olfactory/ frontal cortex, three sections containing temporal and parietal neocortex/thalamus/caudate putamen, one section of cerebral and pons, and one section of brainstem). Histologic sections were cut to 5 micrometers, mounted on positively charged glass slides (Superfrost Plus, Fisher Scientific, Pittsburgh, PA), and replicate sections were routinely stained with hematoxylin and eosin and in situ hybridization (ISH) methods. The progression and severity of central nervous system (CNS) infection in each treatment group was assessed by evaluating the distribution of viral nucleic acids in the brain tissues at each time point. Tissue sections were graded according to the following scale: Grade 0 = no virus-positive cells detected; Grade 1 = rare (less than four) virus-positive cells in the section; Grade 2 = scattered individual cell or very small clusters of virus-positive cells with up to 30 positive cells per section; Grade 3 = numerous individual cells or scattered small clusters of neurons; Grade 4 = large, multifocal clusters of virus-positive cells; and Grade 5 = diffuse areas or zones of virus positive neurons.

In situ hybridization. In situ hybridization was performed with digoxigenin (DIG)-labeled probes as described previously. Positive- and negative-control probes were labeled with DIG-11-dUTP by nick-translation of pPIC9 plasmid (with and without a 3.9-kb VEE viral genome fragment containing the structural protein coding region, respectively) according to the manufacturer’s instructions (Boehringer Mannheim, Indianapolis, IN).

Table 2
Lethality of Venezuelan equine encephalitis virus vaccine strains in C3H/HeN mice

<table>
<thead>
<tr>
<th>Virus</th>
<th>ICLD₅₀⁺</th>
<th>MDD⁺</th>
<th>Sick animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC-83</td>
<td>1.3⁺</td>
<td>12.1⁺</td>
<td>+</td>
</tr>
<tr>
<td>V3526</td>
<td>&gt;6.0⁺</td>
<td>NA</td>
<td>−</td>
</tr>
</tbody>
</table>

* Median intracranial lethal dose.  
⁺ Mean day until death.  
* Log₁₀ plaque forming units per ml.  
NA = not applicable.

Comparative lethality. To compare the relative virulence of V3526, TC-83, and wild-type virus preparations, LD₅₀ was determined in BALB/c mice inoculated IC or IC (Table 1). The ICLD₅₀ for TRD was 0.2 plaque-forming units (PFU) while V3000 was approximately 100 times less lethal, having an ICLD₅₀ of 25.1 PFU. Similarly, the IPLD₅₀ for TRD and V3000 were 6.8 and 30.2 PFU, respectively. The mean day to death (MDD) in IC-inoculated mice was significantly reduced compared to IP-inoculated animals (P < 0.001). Because natural virus populations contain virus subpopulations of varying virality, it is not unexpected that a clone exhibiting less than maximal lethality was selected during the biological and molecular cloning of TRD that resulted in V3000.

In contrast to wild-type viruses, the vaccine strains were highly attenuated in BALB/c mice inoculated IC or IP (Table 1). BALB/c mice given TC-83 IC, however, did consistently exhibit signs of disease (inactivity, rough fur, loss of appetite, and weight loss) but recovered. While physical recovery was complete, TC-83-inoculated mice were subsequently observed to be significantly more aggressive than sham-inoculated controls or V3526-inoculated mice. This aggressive behavior took the form of tail biting cage mates and often progressed to the point where tail amputation was required. This behavior was observed in all TC-83-inoculated mice but was never observed in sham- or V3526-inoculated mice.

With C3H/HeN mice, a more susceptible VEE model, IC inoculation of TC-83 produced a LD₅₀ of 20 PFU, similar to that of wild-type virus in BALB/c mice (Table 2). In contrast, inoculation of C3H/HeN mice with V3526 by the same route was not lethal and did not induce any observable signs of illness at doses up to 10⁶ PFU per animal.

Viremia profiles. We found that viremias in BALB/c mice inoculated SC with TRD peaked at about 10⁶ PFU/mL at 24 hr pi and mean duration was at least 97 hr (Figure 1 and Table 3). In contrast, both V3526 and TC-83 produced much lower titer viremias that peaked at about 200 PFU/mL and averaged only 42 hr and 33 hr duration, respectively. While all mice inoculated with TRD and V3526 produced detectable viremias, only 60% of animals receiving TC-83 became viremic. Surviving mice (all V3526 and TC-83 inoculated mice)
were challenged with $10^5$ PFU of TrD on pi Day 28. All V3526-inoculated mice survived challenge but only the TC-83-inoculated mice that produced detectable viremias survived. TC-83 vaccine failures in both mice and humans were observed previously (Hart MK and Ludwig GV, unpublished data).  

**Replication kinetics after ic inoculation.** To understand the replication kinetics of VEE virus after ic inoculation, mice were inoculated ic with $10^5$ PFU of TrD, V3000, TC-83, or V3526. Brain titers in mice receiving either TrD or V3000 peaked between 24 and 48 hr pi at approximately $10^8$ PFU per g of brain tissue (Figure 2). Brain titers of TrD and V3000 inoculated mice remained at this level until death, which typically occurred between pi Days 5 and 7. TC-83-inoculated mice also produced high titers of virus in the brain, peaking at about $10^8$ PFU per g between 24 and 48 hr pi. Mice inoculated ic with TC-83 did not die and eventually cleared virus from their brains. No infectious virus was detected in these mice after pi day 11. In contrast, V3526 failed to replicate efficiently in the brains of BALB/c mice, producing a brain infection whose titer peaked on pi Day 2 at less than $10^9$ PFU per g of brain. Furthermore, no infectious virus was detected in the brains of V3526-inoculated mice after pi Day 4.

**Reversion studies.** To determine the potential of TC-83 and V3526 to revert to a virulent phenotype in vivo, these viruses were passed in adult mouse brains. Mouse brain titers were consistent throughout the experiment, ranging from between $10^{3.3}$ and $10^{3.9}$ PFU/g for V3526-inoculated mice and from between $10^{4.1}$ and $10^{8.2}$ PFU/g for TC-83-inoculated mice (Table 4). None of the V3526-inoculated mice showed any sign of disease throughout the experiment. In contrast, all TC-83 inoculated mice became ill and one mouse receiv-
ected in a few cells. By pi Day 4, TC-83 virus infection was evident in clusters of Purkinje cells and granular cell neurons in both mouse strains. In the V3526 inoculated mice, large clusters and zones of infected neurons were rarely seen after pi Day 4. In contrast, such zones are common until pi Day 6 in the TC-83-infected BALB/c mice. In C3H/HeN mice, these zones were commonly seen on pi Day 10. These observations are consistent with the higher virus titers seen in TC-83-inoculated mice. To permit a semi-quantitative comparison of the two virus strains, the daily scores of mice with Grades 3, 4, and 5 were totaled (Table 5). These data illustrate the greater overall severity of TC-83 infection observed in BALB/c and C3H/HeN mice inoculated with TC-83. This finding correlates directly with the higher morbidity and mortality seen in that mouse strain. One unexpected finding was the persistence of infected cells in BALB/c mice. Although the numbers of infected cells diminished rapidly after Day 10 in the C3H/HeN mice, in BALB/c mice, V3526-infected cells were present on Day 15, and a few TC-83-infected cells were still observed at 21 days pi. Persistently infected brains did not receive pathology severity rankings over 2 and therefore are not reflected in the data provided in Table 5.

**DISCUSSION**

V3526 represents the first of a new generation of live attenuated alphavirus vaccines. The vaccine offers several distinct advantages over live virus vaccines produced by more traditional procedures such as sequential passage in cultured cells. The new vaccine virus is defined down to the level of its nucleotide sequence and contains multiple, independently attenuating mutations. These characteristics provide the opportunity for greater genetic and phenotypic stability, increased vaccine safety, a more reliable immune response among recipients, and a more consistent product throughout the manufacturing process. In previous work, V3526 was safe and effective in both mice and in non-human primates (Pratt WD, unpublished data) after parenteral inoculation and aerosol administration.

We found that neither V3526 nor TC-83 was neurovirulent after parenteral administration. Subcutaneous inoculation of either virus produced a viremia in BALB/c mice; however, none of the mice developed any neurological signs of disease. The viremia produced by V3526 was more consistent and reliable than that produced by TC-83. Consistent but controlled replication after parenteral vaccination, as evidenced by a viremia, elicited improved immunological responsiveness. In multiple studies, vaccination with V3526 induced seroconversion in 100% of inoculated animals (Ludwig GV, unpublished data). On the other hand, approximately 20% of TC-83 recipients (human, non-human primates, and rodents) fail to develop any detectable immune response. Animals that failed to produce a detectable neutralizing response after TC-83 inoculation were not protected against virus challenge by the parenteral or aerosol routes (Pratt WD, unpublished data; Ludwig GV, unpublished data).

Viremias in TC-83-inoculated mice were delayed relative to those produced by either V3526 or TrD. One possible explanation for this observation is that TC-83 initially replicated poorly after sc inoculation but that selection for, or amplification of more virulent virus subpopulations, or revertants, eventually produced detectable viremia and eventual seroconversion. If the concentration of these subpopulations was relatively low in vaccine preparations, or in the absence of partial reversion, the same phenomenon could help explain the absence of viremia in some animals and the
associated high rate of vaccine failure. Although further research is required to support this hypothesis, similar reversion of attenuated VEE strains in mice has been reported. While TC-83 and V3526 are similar in that neither is neuroinvasive when administered sc (data not shown), they differ in their ability to replicate in nervous tissue after direct ic inoculation. V3526 replicated poorly in the CNS and its replication was limited in both magnitude and cell tropism. Conversely, TC-83 appears to have initially replicated as efficiently as TrD in mouse nervous tissue, producing equivalent brain titers, as seen with wild-type virus. Growth of TC-83 in the CNS was much more rapid and infected more cell types than did V3526. In C3H/HeN mice, TC-83 was universally lethal after ic inoculation, although the day of death was delayed when compared to that of mice inoculated with TrD. While ic inoculation of TC-83 was not lethal in BALB/c mice, the increased magnitude and scope of virus replication in the CNS does appear to lead to increased aggressiveness. We failed to observe any such behavioral changes in mice inoculated with V3526.

Longer-term infection of cells in the CNS with the vaccine strains was unexpected but not unprecedented. Virus RNA was detectable in mouse brains from both TC-83- and V3526-inoculated mice after infectivity in brains dropped below detectable levels. These data are similar to observations reported after ic inoculation of mice with Sindbis virus where viral RNA could be recovered from brains up to 17 months after inoculation. The mechanisms and importance of persistence of alphavirus RNA in nervous tissue are not understood. However, clearance of infectious alphaviruses from nervous tissue can be mediated by antibody-induced restriction of virus gene expression rather than by cytotoxic destruction of virus-infected cells. Therefore, in the absence of viral gene expression, virus-infected neurons could be maintained for extended periods of time.

An essential measure of the safety of a vaccine is based on the relative potential of that vaccine to revert to a virulent phenotype on passage in susceptible cell cultures or host animals. Previous work showed that a precursor to TC-83 (TC-50) easily reverted to virulence in mice inoculated ic. In our studies, we found that V3526 was phenotypically stable after five cell culture passages and during sequential ic passages in mice. Similarly, TC-83 was relatively stable after five mouse brain passages, although it was clearly less attenuated than V3526 in these studies.

Our studies indicate that the rationally derived, genetically engineered VEE vaccine candidate, V3526, is safe, phenotypically stable, and produces consistent viremias in mice after sc vaccination and is significantly less neurovirent than TC-83 after ic inoculation. The candidate performed as well or better than TC-83, in all measures of virulence and stability, and should be considered for further development as a vaccine for human and veterinary use.

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REFERENCES


