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DEVELOPMENT OF A GENETICALLY ENGINEERED
VENEZUELAN EQUINE ENCEPHALITIS VIRUS VACCINE

ANNUAL REPORT

DENNIS W. TRENT

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ABSTRACT

The genomes of the Trinidad donkey (TRD) and TC-83 strains of Venezuelan equine encephalitis (VEE) viruses have been cloned and their nucleotide sequences determined. There are 11 nucleotide differences between the genomes of the TC-83 attenuated virus and its virulent TRD parent strain. One nucleotide substitution and deletion occurred in the 5'- and 3'- noncoding regions of the TC-83 genome, respectively. A conservative Ser to Thr amino acid substitution was identified in nonstructural protein (nsP) three. Two silent mutations, one each in E1 and E2, two amino acid substitutions in E1, and five substitutions in E2 were determined.

The genome of the TRD virus was 11,444 nucleotides long with a 5'- noncoding region of 44 nucleotides. The carboxyterminal portion of VEE nsP3 contained two peptides segments that were repeated. The open-reading-frame of the nsP polyprotein was interrupted by an in-frame opal between nsP3 and nsP4 as has been reported for other alphaviruses. The deduced amino acid sequence of the VEE nsP1, nsP2, nsP3, and nsP4 polypeptide showed 60%-66%, 57%-58%, 34%-44%, and 71%-73% identity with the aligned sequence of cognate proteins of Sindbis and Semliki Forest viruses, respectively.

cDNA encoding the structural proteins of TRD and TC-83 viruses were inserted into vaccinia virus under control of the vaccinia virus 7.5K promoter. Synthesis of the capsid protein and glycoproteins E1 and E2 was demonstrated by immunofluorescence, immunoprecipitation and immunoblotting lysates of recombinant virus infected cells. Mice immunized with the

recombinant VEE/vaccinia virus developed VEE virus-specific neutralizing antibodies and survived intraperitoneal challenge with virulent VEE virus of subtypes IAB, IC, ID, and II. Unlike immunization with attenuated TC-83 virus, mice immunized with the VEE/vaccinia recombinant virus did not survive virulent virus intranasal challenge. T-cell responses of mice and primates to immunization with TC-83 and recombinant vaccinia viruses was serocomplex-specific, with slight cross-reactivity to eastern equine encephalitis virus. Proliferating T-cells secreted interleukin 2 (IL2). Reactivity of primed T-cells with subtype IC, ID, IE, III, and IV viruses was poor and similar to the murine antibody response to immunization. (KT)

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In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health (NIH), Publication No. 85-23, Revised 2985).

The investigator(s) have abided by the National Institutes of Health Guidelines for Research Involving Recombinant DNA Molecules (April 1982) and the Administrative Practices Supplements.

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I. ESTABLISHING A SUBCONTRACT FOR TECHNICAL ASSISTANCE AND FACILITIES
FOR EQUINE TESTING OF THE RECOMBINANT VACCINES.

A contract has been established with Drs. Barry Beaty and Richard Bowen of the Arthropod-Borne Infectious Disease Laboratory at Colorado State University, Fort Collins, Colorado to provide technical assistance for the molecular and immunologic studies to be carried out under the contract. Three positions have been filled with exceptionally qualified individuals. The following have been employed:

<u>Position/Expertise</u>	<u>Employee</u>
Research Associate/Immunology	Allison Johnson
Research Associate/ Molecular Biology	Judith Sneider
Research Associate/DVM	William Short

All individuals received VEE and yellow fever vaccines and began work January 20, 1988.

Horses for experimental vaccine challenge studies have been obtained through a local animal dealer and examined alphavirus antibodies. Eight horses have been identified which are seronegative and suitable for immunization with the experimental vaccines. These animals are currently being held outside prior to being given the first series of vaccinations

with either VEE/vaccinia recombinant, TC-83, or vaccinia virus. The horses will be held in the large animal biocontainment facility for three weeks after vaccination at which time serum samples will be obtained for serological analysis and the animals released outside. On day 42, six weeks after immunization, the horses will be returned to the large animal biocontainment facility to be challenged with equine virulent VEE virus. The animals will be observed two weeks for clinical symptoms, serological analysis, viremia, and then sacrificed for pathological studies. It is anticipated that these studies will be completed in February 1989.

II. VACCINE DEVELOPMENT AND TESTING.

A. Molecular cloning and genetic analysis of the VEE virus genome.

The sequence of VEE TRD and TC-83 genomic RNAs were determined by sequencing cDNA clones. The entire nucleotide and deduced amino acid sequences of the VEE TRD genomic RNA are shown in Fig. 1. Excluding the 5'-m7G cap, the TRD RNA genome was 11,444 nucleotides long with a base composition of 28.2%A, 24.5%C, 25.3%G, and 22.0%U.

In addition to sequence analysis of cDNA clones, three synthetic oligonucleotide primers were used to obtain the 5'- terminal nucleotide sequences from virion RNA templates. Primer extension sequencing of the 5'-termini of the TRD and TC-83 RNAs failed to resolve the 5'-terminal A nucleotide but did confirm the 5'- terminal nucleotides previously determined. TC-83 and TRD viruses differed in this region with a substitution of A for G at genome nucleotide position 3 in the TC-83 RNA. Excluding the 5'-prime cap, the 5'-noncoding region of both genomes was 44 nucleotides in length.

The nucleotide sequence at the 3'-terminal region was obtained by direct sequencing of denatured recombinant plasmid cDNA. These data confirmed the deletion of a U nucleotide in TC-83 virus at genome position 11,405 as previously determined.

Sequencing of the 26S junction region of the 42S RNA where genes encoding the nonstructural polypeptides and those encoding the structural proteins meet revealed the sequences of TRD and TC-83 viruses were identical. The nucleotide difference between TRD and TC-83 reported earlier is in error; the nucleotide position at 7530 is G in both viruses.

Two in-frame AUG codons at nucleotide positions 12-14 and 45-47 occurred within the 5'-terminal 50 nucleotides of the VEE genomic RNA. An out-of-frame AUG codon was present at the 5- terminus in TRD genomic RNAs, but not in TC-83 virus RNA due to substitution of a A nucleotide at position 3. The AXUG sequence at position 45-47 occurred within the consensus sequence CAXXAUGG (where X=any nucleotide) used as initiation codons in eukaryotic mRNA. This sequence was chosen as the initiation codon for the nonstructural VEE virus polyprotein precursor. Translation of the genome was terminated by an ocher (UAA) codon at positions 7524-7526. The deduced translated polyprotein was 2493 amino acids long. The open-reading-frame of the translated region was interrupted by an opal (UGA) codon at nucleotide positions 5682-5684. The in-frame opal codon was present in the genomes of TRD and TC-83 viruses, was also present in the genomes of subtype IAB viruses PTF-39 and 71-180.

Nucleotide sequence data obtained from the cDNA clones (Fig.1) revealed that the genes encoding the nonstructural proteins of VEE TRD and TC-83 viruses differed at nucleotide positions 1873 (T in TRD, A in TC-83), 4698 (A,G), 4809 (T,A), 5877 (G,A), and 7306 (T,C). To determine

if these differences in the cloned cDNA accurately represented nucleotide differences between the two viruses, sequencing of TRD and TC-83 42S RNA templates was performed with synthetic oligonucleotide primers. Nucleotide positions 1873, 4698, 5877, and 7306 were confirmed to be A, G, G, and U, respectively. Thus, the T-A nucleotide difference at position 4809, encoding a Ser to Thr amino acid change, was the only confirmed difference between the genes encoding the nonstructural proteins of the TRD and TC-83 viruses. This mutation occurred in nsP3 at amino acid position 260. The other nucleotide and deduced amino acid sequences of TRD shown in Fig. 1 reflect the corrections obtained by primer extension sequencing of the virion RNA.

The putative cleavage sites used to generate the nonstructural polypeptides from the polyprotein precursor were identified by comparing the VEE virus amino acid sequence with the sequences of those for Sindbis (SIN) and Simliki Forest (SF) viruses. The amino acid sequences of the translated nonstructural polyproteins of TRD, SIN, and SF viruses were aligned to provide maximal homology and the sequences of the cognate proteins compared. The nsP1, nsP2, nsP3, and nsP4 polypeptides of VEE TRD virus showed 60%-66%, 57%-58%, 35%-44%, and 73%-71% sequence identity, respectively. The nsP4 protein was the most highly conserved of the four nonstructural proteins. When the nucleotide sequences of the translated regions of these viruses were aligned, VEE sequences encoding nsP1, nsP2, and nsP4 showed 60%-63%, 57%-58%, 44%-49%, and 65%-63% identity, respectively, with cognate genes of SIN and SF viruses.

The deduced molecular weights of the nsP1, nsP2, and nsP4 polypeptides were approximately 60, 89, and 68-69 kd, respectively for VEE, SIN, and SF viruses. The 52 kd nsP3 protein of VEE virus encoded at

positions 330-350, was highly conserved when compared to SIN and SF viruses. For example, 94% of 18 Trp, 73% of 73 Phe, 70% of 61 Cys, 70% of 120 Gly, and 69% of 116 Pro residues were conserved in the aligned nsP sequences of SIN and SF viruses.

The nsP3 polypeptide, which was the least conserved of the four nonstructural proteins, has some interesting features. Although the amino-terminal half of the molecule is highly conserved among VEE, SIN, and SF viruses, the carboxyl-terminal half of VEE nsP3 showed virtually no sequence identity with the same region of SIN and SF nsP3 polypeptides. The carboxy-terminal portion of VEE nsP3 contained a short repeated peptide segment (PXPAPRT, where X= a variable amino acid) and a long 34 amino acid sequence repeat: TPSXXPSRXXSRTSLVSPPGVNRVITREEXEXX. These repeated peptide segments were identical in TRD and TC-83. SF virus nsP3 has a repeated peptide segment that is nearly identical to the first short repeated peptide of VEE virus. Although the carboxyl portion of the SF virus nsP3 peptide shows some identity to that of SIN virus, this region of SF virus nsP3 lacked 70 and 73 amino acids relative to VEE and SIN viruses, respectively. The in-frame opal termination codon (asterisk in Fig. 1) located six amino acid residues upstream from the carboxyl-terminus of VEE nsP3 was aligned with the in frame opal codon reported for SIN virus.

The nucleotide and deduced amino acid sequence differences between the genomes of VEE TRD and TC-83 virus are summarized in Table 1. TC-83 virus contained a single mutation in the 5'- noncoding region. Additional mutations were noted in the E1 and E2 glycoproteins, and in the 3'-noncoding region.

B. Construction of an infectious VEE virus cDNA clone.

Nucleotide sequencing of 11 cDNA clones encoding the entire 42S RNA genomes of both TRD and TC-83 viruses has facilitated the development of a strategy for assembly of a single clone encoding the entire genomes of both viruses (Fig. 2). Site-specific mutagenesis has been used to create three silent mutations in the 3'-end of TC83 clone pTC-46. To serve as identification markers for future cDNA-derived virus populations. The 5'-prime end of clone pTC46 as mutagenized to contain the bacteriophage T7 promoter to control transcription of full-length 42S RNA from the linear double-stranded full-length cDNA clone. An XbaI cloning site has been inserted into the clone to facilitate final assembly of the full-length cDNA. cDNA regions of clones pTC-19, pTRD-30, and pTC-9 have been spliced together to obtain a 3950-bp clone. Clone pTRD-26 has now been added to the 3950-bp pTC30/19/9 clone to complete assembly of the middle section of the cDNA.

At the 3'-end of the genome, clone pTC-5 which encodes the structural proteins of TC-83 virus, lacks the 3'-terminal 65 nucleotides of the 3'-noncoding region. This clone is currently being modified. To do this, the clone has been inserted into the single-stranded M13 phage and is undergoing modifications to construct the 3'-end of the genome. Once this task has been completed, the middle region, 5'-end, and 3'-end clones will be ligated together to form the final full-length cDNA.

C. Expression of VEE virus structural genes by vaccinia virus recombinant.

Three vaccinia/VEE recombinant viruses were produced: VAAC/TC-5A, expressing the genes encoding the structural proteins of VEE TC-83 virus, and two vaccinia/TRD virus recombinant, VACC/TRD-1A and VACC/TRD-20A, expressing the structural proteins of VEE TRD virus. To confirm insertion of VEE cDNA into the vaccinia thymidine gene, DNA purified from wild-type or recombinant vaccinia virus was digested with Hind III for southern blot hybridization. The 5 kb Hind III J fragment of vaccinia virus was not present in the recombinant virus DNA however, a new band of about 9 kb was present in the gel pattern. This new fragment is of the predicted size and mobility of the Hind III J fragment which contains the VEE cDNA fragment plus the 7.5 K promoter from the chimeric plasmid.

Immunoblotting of VACC/TC-5A, VACC/TRD-1A and VACC/TRD-20A virus infected cell lysates clearly showed a polypeptide band that comigrates with the VEE virus capsid protein. Lysates of cells infected with the three recombinant viruses also contained polypeptides that comigrated with the E1 and E2 envelope glycoproteins. From these experiments it was concluded that the structural proteins of VEE virus were synthesized and processed in cells infected with the vaccinia recombinant viruses.

Expression of VEE proteins in recombinant virus-infected cells was also demonstrated by fluorescent antibody (FA) analysis. VEE virus antigens were detected in acetone-fixed VEE TC-83 or recombinant VACC/TC-5A virus infected cells. Although the level of VEE structural protein expression in the recombinant virus-infected cells was usually lower than in cells infected with VEE virus, many of the recombinant

virus-infected cells showed an FA intensity equal to that of the TC-83 virus infected cells. Surface expression of VEE virus antigens as indicated by FA analysis of unfixed cells was lower in the recombinant virus-infected cells.

Antiserum prepared against vaccinia virus showed positive FA only with vaccinia and VACC/VEE virus-infected cells. Polyvalent anti-TC-83 virus ascitic fluid reacted with cells infected with VEE virus or VACC/VEE recombinant virus (Table 2). The availability of well characterized monoclonal antibodies identifying epitopes in the E1 and E2 glycoproteins of VEE virus permitted us to investigate the expression of VEE-specific antigenic determinants. The monoclonal anti-E2 monoclonal antibodies shown in Table 2 generally reacted similarly with cells infected with VEE or VACC/VEE virus. In particular, the TC-83-specific antibody 5B4D-6 (epitope E2a) reacted more strongly with recombinant VACC/TC-5A infected cells than with VACC/TRD-1A infected cells. Monoclonal antibodies 1A4A-1 (E2c), 1A4D-1 (E2f), and 1A3A-9 (E2g) showed positive FA reactivities with cells infected with both VEE virus and the recombinant. Although antibody 1A3A-5 (E2e) reacted poorly with recombinant virus infected cells, a pattern of TC-83 antigen similar to that seen with VEE virus-infected cells was observed. Antibody 1A6C-3 (E2d), which reacted poorly with cells expressing TC-83, TRD or VAC/TRD-1A virus antigens, failed to react with VACC/TC-5A infected cells. The 1A3B-7 (E2h) antibody failed to react well with VACC/TRD-1A recombinant-infected cells, while reacting strongly with cells infected with VACC/TC-5A, TC-83, or TRD virus.

Of the four E1 glycoprotein epitopes identified for VEE virus, only E1b and E1d epitopes were detected in recombinant virus-infected cells

(Table 1). Although the Ela and Elc epitopes were detected in VEE TC-83 and TRD virus-infected, acetone-fixed cells, these two epitopes were not evident by FA in recombinant VACC/TC-5A or VACC/TRD-1A infected cells.

D. Humoral and cell-mediated immune responses to the vaccinia/VEE recombinant.

1. Murine humoral responses to immunization.

Responses of AJ mice to immunization with recombinant VACC/TC-5A virus are shown in Table 3. Mice were immunized i.p. with 10^{-4} or i.d. with 10^{-8} PFU of VACC virus or 10^5 to 10^7 PFU of recombinant vaccinia virus VACC/TC-5A. Animals were bled 3 weeks later to determine prechallenge antibody titers and again two weeks after virulent TRD virus challenge. Only those mice receiving TC-83 or VACC/TC-5A vaccine virus were protected from virulent TRD virus challenge. Mice which received 10^5 or 10^7 PFU of VACC/TC-5A virus developed similar levels of prechallenge HI or ELISA antibody. Animals which were given 10^5 PFU of VACC/TC-5A however, developed no measurable or much reduced HI or ELISA antibody. The development of vaccinia-induced tail blister, survival to TRD virus challenge, and antibody response were dose-related in the animals immunized with VACC/TC-5A virus. Reliable immunity was obtained only in animals given a VACC/TC-5A dose of 10^7 PFU. The attenuated TC-83 virus elicited much higher antibody titers in mice. Although post-challenge VEE virus viremias were not determined, post-challenge antibody titers showed a dose-related boost in VACC/TC-5A immunized animals that was much greater than that shown by the TC-83 mouse

group (Table 1). This suggested that the apparent lower level of immunity in recombinant-immunized mice was less effective in inhibiting replication of the challenge virus.

Prechallenge Nt antibody titers in individual animals receiving various doses of recombinant vaccine showed a dose-related response (Table 4). In all cases, the response to the vaccinia recombinant was lower than that seen in animals which received the TC-83 vaccine. Three mice immunized with the recombinant did not develop Nt antibody and did not survive virus challenge.

AJ mice, immunized with 10^7 PFU of VACC/TC-5A virus, were bled at 18, 33, and 86 days post-immunization to determine the short-term duration of VEE virus immunity (Table 5). Neutralizing antibody titers indicated that antibody levels elicited by the VACC/TACF5A vaccine were stable for at least 3 months.

Neutralization antibody titers were determined for both Swiss NIH and C3H mice that received 10^8 PFU of VACC/TC-5A virus i.d. The recombinant VACC/TC-5A vaccine elicited a weaker immune response in outbred Swiss NIH mice than in the inbred C3H or AJ mice. The VACC/TAC-5A induced tail lesions were also reduced in Swiss NIH mice as compared to either C3H or the AJ mice.

2. Murine cross-protection studies.

Five individual, high-titered anti-anti-TC-83 or anti-VACC/TC-5A mouse sera were pooled and tested for their ability to neutralize different antigenic subtypes of VEE virus (Table 6). The Nt cross reactivities of anti-TC-83 and anti-Vacc/TC-5A were very similar.

Both sera neutralized epizootic VEE viruses 1AB and 1C efficiently. Subtype 1D, 1E, and 2 viruses were neutralized only at high antibody concentrations. Anti-VACC/TC-5A serum Nt titers were lower than those of anti-TC-83 serum. Neither TC-83 nor VACC/TC-5A antiserum neutralized VEE subtype 4 or Western equine encephalitis virus.

In vitro neutralization test information indicated that the recombinant VACC/TC-5A vaccine should protect mice against challenge with subtype 1A-D viruses (Table 6). To evaluate this, C3H and NIH Swiss mice were immunized with TC-83 or recombinant virus and challenged with VEE TRD virus (Table 7). Both TC-83 and VACC/TC-5A vaccine viruses protected mice against lethal infection with 1AB, 1C, and 1D virus infection. Although protection of Swiss NIH mice against infection with subtype 2 virus challenge was ambiguous, the C3H mice were clearly protected by both vaccines from challenge with subtype 2 virus. High-doses of TRD and subtype 1C viruses caused three fatalities in recombinant-immunized, but none in TC-83-immunized, Swiss NIH mice (Table 7).

To determine the level of protection afforded by immunization with recombinant vaccinia virus to aerosol infection, mice were immunized with VACC/TC-5A virus and challenged with virulent TRD virus. The VACC/TC-5A vaccine effectively protected mice against peripheral infection but not intranasal challenge with TRD virus. However, the TC-83 vaccine protected mice from challenge virus given by either route (Table 8). Five of twenty TC-83-immunized C3H mice succumbed to intranasal TRD virus challenge. The six VACC/TC-5A-immunized mice which survived intranasal challenge all became ill

after challenge, lost weight, but showed no signs of paralysis. The six AJ survivors recovered very slowly and were not normal 3 weeks post challenge.

3. Murine helper T-cell responses to VEE vaccine candidates.

Previous investigations in our laboratory have characterized the response of splenic murine T lymphocytes to VEE virus immunization. Proliferating primed lymphocytes were identified as helper T-cells by immune complement depletion or florescent-activated cell scanning with monoclonal antibody typing reagents. The proliferative in vitro response to VEE virus priming was VEE serocomplex specific, with slight cross-reactivity to EEE virus, and peaked at 14 days post-inoculation. Proliferating cells secreted interleukin 2 (IL2). Considerable activity could be identified as late as 56 days post-immunization. Animals immunized with TC-83 vaccine demonstrated a good T-helper cell response to VEE subtypes IAB and II viruses. Reactivity of primed T-cells with subtypes IC, ID, IE, III and IV viruses was poor. The cross-reactivity of the T-helper cell response was therefore similar to the murine antibody response to VEE virus immunization.

Investigations of murine helper T-cell response to TC-5A has been completed. In all cases, TC-5A immunized mice demonstrate an authentic helper T-cell response after a single inoculation of a high or low dose of immunogen. The responding cell population was Thy1^+ , L3T4^+ , Lyt 1.1^+ , and Lyt 2.1^- , and secreted IL2 in response to antigen. The specificity of the proliferative response was similar to that shown with TC-83 vaccine as described previously.

4. Primate antibody response VEE vaccine candidates.

Two groups of primate experiments have now been completed. Animals in the first animal group were inoculated intradermal with 100,000 pfu of TC-5A virus. Control animals were immunized with TC-83 or C-84 vaccines, or vaccinia. Immunized animals were monitored for antibody production, and peripherally challenged with 400,000 pfu of the virulent IB subtype virus, 71-180. Animals in the second group were similarly immunized, but challenged intranasally with 44,000 pfu of 71-180 virus. Four of 6 TC-5A immunized animals developed virus neutralizing antibody titers. When these animals were challenged with virus, 1 of 3 peripherally challenged animals demonstrated a low level transient viremia, while 1 of 3 intranasally challenged animals developed a significant viremia. Animal viremia exactly correlated with lack of neutralizing antibody. Leukopenia was observed only in the intranasally challenged, viremic animal.

All animals immunized with control vaccines (TC-83 and C-84) developed neutralizing antibodies. One C-84 immunized animal developed significant leukopenia and viremia following intranasal challenge. All TC-83 immunized animals were solidly protected. All TC-5A and vaccinia immunized controls developed vaccinia neutralizing antibodies. All vaccinia immunized and non-immunized control animals developed significant viremia and leukopenia following VEE virus challenge.

Because the virus challenge is non-lethal, post-challenge antibody levels were monitored. Four of 6 TC-5A immunized animals demonstrated an anamnestic response. Two animals had stable antibody

titers. Three of 6 C-84 immunized animals demonstrated an anamnestic response. The anamnestic responses could be due to low level virus replication following challenge, however it was not possible to correlate low level pre-challenge neutralization titers to subsequent secondary antibody response. No TC-83 immunized animals demonstrated an anamnestic response. The lack of a secondary antibody response in TC-83 immunized animals was probably due to limited viral replication occurring in TC-83 immunized animals.

We attempted to identify neural invasion in intranasally challenged animals by monitoring IgM and IgG antibody levels in cerebrospinal (CSF) fluid. IgM antibody could be detected in only the vaccinia immunized controls. IgG antibody was detected in 2 of 3 TC-5A vaccinated animals and 1 of 3 C-84 vaccinated animals. Serum IgG and IgM titers are currently being determined.

5. Helper T-cell responses in vaccinated primates.

Peripheral blood mononuclear cells were obtained from immunized animals. In general, the frequency of antigen primed T-cells in peripheral blood was too low to use this as a lymphocyte source. We subsequently tested lymphocyte populations from axillary lymph nodes of vaccinated, challenged animals. The axillary lymph node appears to be an adequate lymphocyte source.

Antigen specific helper T-cell activity could be defined by both in vitro proliferation and IL2 production. The antigen specificity of this response was similar to that previously described in the murine model. Fluorescent activated cell scanning of challenged

animals demonstrated no difference in comparative population levels of cytotoxic and helper T-cell subsets. We are currently identifying antigen-specific T-helper cells from axillary lymph nodes of immunized animals prior to virus challenge.

III. DISCUSSION.

Attenuation of a virulent virus may result from mutations in the virus structural proteins which affect viral morphogenesis, host cell tropisms, and interactions with the host-immune system. Comparisons of the entire genomes of TRD and TC-83 viruses reveals only 11 nucleotide changes (0.1%) in 11,444 nucleotide genome of the virus. The total number and distribution of TC-83 mutations contrasts with the 17D yellow fever virus vaccine in which 68 nucleotide mutations occurred throughout the genome. A single nucleotide mutation in the 5'- and 3'- noncoding regions of TC-83 virus confirm earlier studies which identified these nucleotide changes. Sequence analysis of mouse-virulent and mouse-avirulent strains of Ross River virus revealed 284 nucleotide differences between the genomes of the two viruses as well as deletions or insertions in the noncoding regions. Because multiple substitutions occurred in the translated structural and nonstructural regions throughout the genome, identification of specific mutations involved in virulence is seriously compromised.

Mutations in the noncoding regions and a conservative Ser to Thr substitution in nsP3 of TC-83 virus may contribute to attenuation, the dearth of mutations in the 5'-two-thirds of the genome enhances the significance of changes in the envelope glycoprotein genes. The nonconservative nature of the five amino acid substitutions in E2 makes it

a prime target as a determinant of virulence. Reversion of a mutation which affects the isoelectric point of E1 in a temperature-sensitive, avirulent mutant of VEE virus occurred concomitantly with reversion to virulence. Specific loci in both E1 and E2 seem to be important in SIN virus neurovirulence in adult mice. Studies with poliovirus infectious cDNA clones indicate that loci involved in attenuation are distributed in several genes and alterations in antigenicity do not correlate with virulence changes.

Loss of envelope protein epitopes involved in neutralization correlate with attenuation of rabies virus, however, such changes are not involved in VEE virus attenuation. Of the 12 epitopes identified on the envelope glycoproteins of VEE virus, only E2a is TC-83 virus specific. This epitope is defined by a monoclonal antibody that does not inhibit hemagglutination or neutralize virus infectivity. Eleven epitopes, including those involved in virus neutralization, are conserved in both TRD and TC-83 viruses.

We have inserted cDNA encoding the VEE TRD and TC-83 virus structural polyprotein precursor into the thymidine kinase gene of vaccinia virus. The vaccinia/VEE recombinant virus directs the synthesis of a polyprotein which is cleaved to form VEE virus structural proteins which have the appropriate size and react with specific antibodies. Direct immunofluorescence tests using monoclonal antibodies confirm expression of envelope glycoprotein epitopes E2a, E2c, E2f, E2g, E1b, and E1d. The reduced expression of epitope E2d by the vaccinia recombinant is unexplained. Expression of the E2c epitope is especially important because it defines the critical neutralization site on the VEE virus particle. Other epitopes on the envelope glycoproteins which are thought

to contribute to virus neutralization are expressed by the recombinant virus. Epitopes E2c and E2h, which are conserved on the E2 glycoprotein of subtype 1AB, 1C, 1D, and 2 viruses are expressed by the recombinant virus provide protection from heterologous VEE virus challenge.

Recombinant VACC/TC-5A induced immunity in outbred Swiss NIH mice was weaker than in the inbred AJ or C3H mice. This differential response of the TC-83 and VACC/TC-SA vaccines in different mouse strains was expected. VEE virus replicates in most tissues of the mouse and therefore, the TC-83 vaccine would stimulate the immune system in many body regions. Replication of TC-83 vaccine virus in the nasopharynx would be expected to stimulate local secretory immunity, and provide immunity to intranasal challenge with TRD virus in the TC-83 immunized mice. Although passive monoclonal Nt antibody protects mice from peripheral virus challenge, it does not protect mice from intranasal challenge. In this respect, immunity elicited by the recombinant was like immunization with formaldehyde-inactivated C-84 VEE virus. These results raise questions concerning the route of immunization and perhaps the choice of vaccinia virus strains used for gene expression. A more virulent vaccinia strain may be an appropriate veterinary vaccine, however, it would most likely be unsuitable for human use.

IV. PUBLICATIONS.

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Kinney, R.M., Esposito, J. J., Johhson, B.J.B., Roehrig, J.T., Mathews, J.H., Barraett, A.D., and Trent, D.W. (1988). Recombinant vaccinia/Venequelan equine encephalitis (VEE) virus expresses VEE structural proteins. J. Gen. Virol. 69: (in press).

Kinney, R.M., Johnson, B.J.B., Welch, J.B., Tsuchiya, K.R., and Trent, D.W. (1989). The full-length nucleotide sequence of the virulent Trinidad donkey strain of Venezuelan equine encephalitis virus and its attenuated vaccine derivative, strain TC-83. Virology (in press).

Table 1. Nucleotide and Deduced Amino Acid Differences Between
VEE TRD and TC-83 Viruses

<u>Position</u>		<u>Nucleotide</u>		<u>Amino Acid Change</u>	
<u>Nucleotide</u>	<u>Amino Acid</u>	<u>TRD</u>	<u>TC-83</u>	<u>TRD</u>	<u>TC-83</u>
3	5'-noncoding	G	A	Noncoding	
4809	nsP3-260	U	A	Ser	Thr
8584	E2-7	G	U	Lys	Asn
8816	E2-85	C	U	His	Tyr
8922	E2-120	C	G	Thr	Arg
9138	E2-192	U	A	Val	Asp
9397	E2-278	U	C	None	
9450	E2-296	C	U	Thr	Ile
10478	E1-161	U	A	Leu	Ile
10630	E1-211	A	U	None	
11405	3'-noncoding	UU	U	Noncoding	

Table 2. Indirect immunofluorescence of acetone-fixed CV-1 cells
24 h post-infection

Antibody*	Specificity	Virus					
		Unin- fected	Vaccinia	VACC/ TC-5A	VACC/ TRD-1A	TC-83	TRD
Mouse serum	Vaccinia	-	3†	3	3	-	-
MHIAF	VEE TC-83	-	-	3	3	4	4
5B4D-6	E2 ^a TC-83	-	-	3	1	4	1
1A4A-1	E2 ^c PTF-39	-	-	3	3	4	4
1A6C-3	E2 ^d EVE	-	-	-	2	2	2
1A3A-5	E2 ^e P676	-	-	2	-	4	2
1A4D-1	E2 ^f TRD	-	-	3	3	4	4
1A3A-9	E2 ^g PTF-39	-	-	3	3	4	4
1A3B-7	E2 ^h PTF-39	-	-	3	1	4	4
3B2D-5	E1 ^a TC-83	-	-	-	-	3	3
3B2A-9	E1 ^b TC-83	-	-	3	3	3	3
5B6A-6	E1 ^c TC-83	-	-	-	-	3	1
3A5B-1	E1 ^d TC-83	-	-	3	3	4	4

*Alphanumeric designations are anti-VEE virus monoclonal antibodies (mouse ascitic fluids). Epitope specificity and virus used to elicit monoclonal antibody are shown. MHIAF = mouse hyperimmune ascitic fluid. All antibody preparations were tested at 1:300 dilution.

†Relative fluorescence as determined by two observers in at least two experiments. - = negative; 4 = maximum.

TABLE 3. HI and ELISA serum titers in A/J mice immunized with TC-83 or recombinant VACC/TC-5A virus

Vaccine		Log ₁₀ Geometric Mean Titer ($\pm$ S.E.) ^d							
Virus	Dose ^a (PFU)	Challenge ^b	Survival ^c	Prechallenge		Postchallenge		Ratio ^e	
				HI	ELISA	HI	ELISA	HI	ELISA
TC-83	10 ⁴	TRD	8/8	2.47 (0.09)	$\geq$ 5.01	3.03 (0.14)	N.D.	4	N.D. ^f
VACC/TC-5A	10 ⁷	-	8/8	1.64 (0.13)	3.17 (0.13)	1.94 (0.09)	3.24 (0.14)	2	1
VACC/TC-5A	10 ⁷	TRD	8/8	1.56 (0.09)	3.24 (0.14)	2.77 (0.17)	4.67 (0.22)	16	29
VACC/TC-5A	10 ⁶	TRD	7/8	1.43 (0.04)	3.03 (0.07)	2.72 (0.13)	4.75 (0.09)	19	53
VACC/TC-5A	10 ⁵	TRD	5/8	1.18 (0.03)	2.36 (0.06)	3.10 (0.16)	5.01 (0.10)	85	445
VACC	10 ⁸	TRD	0/8	<1.0	<1.0	-	-	-	-
PBS	-	TRD	0/8	<1.0	<1.0	-	-	-	-

^aPFU determined in Vero cells. TC-83 and VACC viruses given by intraperitoneal injection or tail scarification, respectively.

^bChallenge = 100 IPLD₅₀ (15 PFU) of TRD virus.

^cNumber of survivors/number in group.

^dDetermined from individual titers of five (10⁵ PFU VACC/TC-5A), seven (10⁶ PFU VACC/TC-5A), or eight (all others) mice per group.

^ePostchallenge GMT/prechallenge GMT (GMT = geometric mean titer).

^fN.D. = not done.

TABLE 4. VEE neutralizing antibody titers in
A/J mice 3 weeks after immunization
with VACC/TC-5A virus

Mouse Number	Vaccine dose (PFU)		
	10 ⁷	10 ⁶	10 ⁵
1	2,560 ^a	160	10
2	640	<10 ^b	10
3	320	80	<10 ^b
4	640	<10	<10 ^b
5	5,120	320	20
6	80	320	160
7	80	20	<10 ^b
8	20	320	20

^aReciprocal of highest antiserum dilution
that inhibited 70% or more of the VEE TRD
virus (60 PFU) used in the test.

^bMouse died after challenge with
100 IPLD₅₀ of VEE TRD virus.

TABLE 5. Duration of immunity in individual
A/J mice immunized intradermally with
 10^7 PFU of recombinant VACC/TC-5A
virus

Mouse	Days postimmunization		
	18	33	86
1	320 ^a	2,560	1,280
2	640	160	320
3	2,560	640	320
4	640	2,560	2,560
5	5,120	5,120	10,240
6	20	80	40

^aReciprocal of highest antiserum dilution
that inhibited 70% or more of VEE TRD virus
(60 to 80 PFU) used in neutralization test.

TABLE 6. Cross-reactivities of sera pooled from A/J mice immunized with VEE TC-83 or recombinant VACC/TC-5A virus.

Virus	Pooled mouse serum	
	VACC/TC-5A	TC-83
VACC	80 ^a	<10
VACC/TC-5A	160	<10
TRD (1AB) ^b	1,280	≥20,480
P676 (1C)	320	≥640
3880 (1D)	10	160
Mena II (1E)	10	80
EVE (2)	40	320
PIX (4)	<10	<10
WEE	<10	<10

^aEndpoint = 70% neutralization of 60 to 90 PFU.

^bVEE subtype.

TABLE 7. VACC/TC-5A and TC-83 vaccine efficacy in protecting C3H or Swiss NIH mice from intraperitoneal challenge with four VEE virus strains

Challenge		Survival ^a			
Virus	Dose (PFU)	VACC/TC-5A		TC-83	
		C3H	Swiss	C3H	Swiss
TRD (1AB) ^b	6 X 10 ¹	6/6 ^c	6/6	6/6	6/6
	6 X 10 ²	6/6	6/6	6/6	6/6
	6 X 10 ³	6/6	5/6	6/6	6/6
	6 X 10 ⁵	6/6	5/6	6/6	6/6
P676 (1C)	1.1 X 10 ⁴	6/6	6/6	6/6	6/6
	1.1 X 10 ⁶	6/6	5/6	6/6	6/6
3880 (1D)	1 X 10 ¹	6/6	6/6	6/6	6/6
	1 X 10 ³	6/6	6/6	6/6	6/6
EVE (2)	1 X 10 ⁸	8/8	8/8	8/8	8/8

^aAll mice in 17 PBS control groups (nine virus challenge doses, two mouse strains) succumbed to virus challenge. Only three of eight PBS control Swiss NIH mice died after EVE virus challenge.

^bVEE subtype.

^cNumber of survivors/total.

TABLE 8. Summary of survival of VACC/TC-5A- or TC-83-immunized mice challenged intraperitoneally or intranasally with virulent VEE TRD virus

Mouse strain	<u>Intraperitoneal challenge^a</u>		<u>Intranasal challenge^b</u>	
	VACC/TC-5A	TC-83	VACC/TC-5A	TC-83
A/J	42/42	8/8	6/24	16/16
C3H	30/30	30/30	0/30	15/20
Swiss NIH	22/24	24/24	1/30	18/18

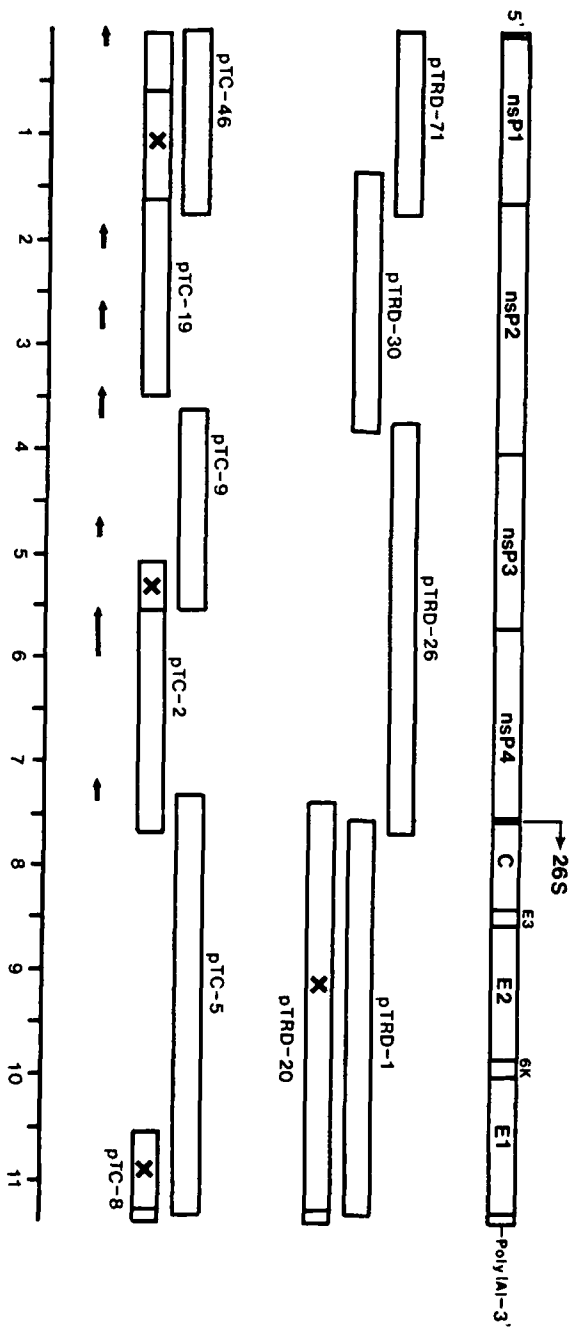
^aChallenge dose = 15 PFU to 6×10^5 PFU of TRD virus.

^bChallenge dose = 3,300 (A/J) or 10,500 (C3H and Swiss NIH) PFU of TRD virus.

FIGURE LEGEND

Figure 1. Strategy for obtaining the complete nucleotide sequences of VEE TRD and TC-83 viruses. cDNA clones that were sequenced are indicated by open rectangles lacking the 'X' symbol. Arrows indicate the extent of primer extension RNA sequencing. The organization of the genome is shown at the top, and genome position in kilobases is shown at the bottom.

Figure 2. Nucleotide sequence of the VEE TRD 42S genomic RNA and the deduced amino acid sequence of the encoded polypeptides. The single letter abbreviation is aligned with the first nucleotide of codon. Arrows indicate proteolytic cleavage sites of the translated polyproteins and the putative 26S mRNA start site. The overlined amino acid sequences represent probable transmembrane domains of the E1 and E2 envelope glycoproteins. Amino acid repeats in nsP3 are outlined. The in-frame opal termination codon near the carboxyl terminus of nsP3 (*) and the confirmed nucleotide mutations (+) in TC-83 virus are indicated. Potential glycosylation sites in E1, E2, and E3 are indicated by solid circles.



nsP4

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76 77 78 79 80 81 82 83 84 85 86 87 88 89 90 91 92 93 94 95 96 97 98 99 100 101 102 103 104 105 106 107 108 109 110 111 112 113 114 115 116 117 118 119 120 121 122 123 124 125 126 127 128 129 130 131 132 133 134 135 136 137 138 139 140 141 142 143 144 145 146 147 148 149 150 151 152 153 154 155 156 157 158 159 160 161 162 163 164 165 166 167 168 169 170 171 172 173 174 175 176 177 178 179 180 181 182 183 184 185 186 187 188 189 190 191 192 193 194 195 196 197 198 199 200 201 202 203 204 205 206 207 208 209 210 211 212 213 214 215 216 217 218 219 220 221 222 223 224 225 226 227 228 229 230 231 232 233 234 235 236 237 238 239 240 241 242 243 244 245 246 247 248 249 250 251 252 253 254 255 256 257 258 259 260 261 262 263 264 265 266 267 268 269 270 271 272 273 274 275 276 277 278 279 280 281 282 283 284 285 286 287 288 289 290 291 292 293 294 295 296 297 298 299 300 301 302 303 304 305 306 307 308 309 310 311 312 313 314 315 316 317 318 319 320 321 322 323 324 325 326 327 328 329 330 331 332 333 334 335 336 337 338 339 340 341 342 343 344 345 346 347 348 349 350 351 352 353 354 355 356 357 358 359 360 361 362 363 364 365 366 367 368 369 370 371 372 373 374 375 376 377 378 379 380 381 382 383 384 385 386 387 388 389 390 391 392 393 394 395 396 397 398 399 400 401 402 403 404 405 406 407 408 409 410 411 412 413 414 415 416 417 418 419 420 421 422 423 424 425 426 427 428 429 430 431 432 433 434 435 436 437 438 439 440 441 442 443 444 445 446 447 448 449 450 451 452 453 454 455 456 457 458 459 460 461 462 463 464 465 466 467 468 469 470 471 472 473 474 475 476 477 478 479 480 481 482 483 484 485 486 487 488 489 490 491 492 493 494 495 496 497 498 499 500 501 502 503 504 505 506 507 508 509 510 511 512 513 514 515 516 517 518 519 520 521 522 523 524 525 526 527 528 529 530 531 532 533 534 535 536 537 538 539 540 541 542 543 544 545 546 547 548 549 550 551 552 553 554 555 556 557 558 559 560 561 562 563 564 565 566 567 568 569 570 571 572 573 574 575 576 577 578 579 580 581 582 583 584 585 586 587 588 589 590 591 592 593 594 595 596 597 598 599 600 601 602 603 604 605 606 607 608 609 610 611 612 613 614 615 616 617 618 619 620 621 622 623 624 625 626 627 628 629 630 631 632 633 634 635 636 637 638 639 640 641 642 643 644 645 646 647 648 649 650 651 652 653 654 655 656 657 658 659 660 661 662 663 664 665 666 667 668 669 670 671 672 673 674 675 676 677 678 679 680 681 682 683 684 685 686 687 688 689 690 691 692 693 694 695 696 697 698 699 700 701 702 703 704 705 706 707 708 709 710 711 712 713 714 715 716 717 718 719 720 721 722 723 724 725 726 727 728 729 730 731 732 733 734 735 736 737 738 739 740 741 742 743 744 745 746 747 748 749 750 751 752 753 754 755 756 757 758 759 760 761 762 763 764 765 766 767 768 769 770 771 772 773 774 775 776 777 778 779 780 781 782 783 784 785 786 787 788 789 790 791 792 793 794 795 796 797 798 799 800 801 802 803 804 805 806 807 808 809 810 811 812 813 814 815 816 817 818 819 820 821 822 823 824 825 826 827 828 829 830 831 832 833 834 835 836 837 838 839 840 841 842 843 844 845 846 847 848 849 850 851 852 853 854 855 856 857 858 859 860 861 862 863 864 865 866 867 868 869 870 871 872 873 874 875 876 877 878 879 880 881 882 883 884 885 886 887 888 889 890 891 892 893 894 895 896 897 898 899 900 901 902 903 904 905 906 907 908 909 910 911 912 913 914 915 916 917 918 919 920 921 922 923 924 925 926 927 928 929 930 931 932 933 934 935 936 937 938 939 940 941 942 943 944 945 946 947 948 949 950 951 952 953 954 955 956 957 958 959 960 961 962 963 964 965 966 967 968 969 970 971 972 973 974 975 976 977 978 979 980 981 982 983 984 985 986 987 988 989 990 991 992 993 994 995 996 997 998 999 1000 1001 1002 1003 1004 1005 1006 1007 1008 1009 1010 1011 1012 1013 1014 1015 1016 1017 1018 1019 1020 1021 1022 1023 1024 1025 1026 1027 1028 1029 1030 1031 1032 1033 1034 1035 1036 1037 1038 103