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# Novel vaccine against Venezuelan equine encephalitis combines advantages of DNA immunization and a live attenuated vaccine

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ABSTRACT

DNA vaccines combine remarkable genetic and chemical stability with proven safety and efficacy in animal models, while remaining less immunogenic in humans. In contrast, live-attenuated vaccines have the advantage of inducing rapid, robust, long-term immunity after a single-dose vaccination. Here we describe novel iDNA vaccine technology that is based on an infectious DNA platform and combines advantages of DNA and live attenuated vaccines. We applied this technology for vaccination against infection with Venezuelan equine encephalitis virus (VEEV), an alphavirus from the *Togaviridae* family. The iDNA vaccine is based on transcription of the full-length genomic RNA of the TC-83 live-attenuated virus from plasmid DNA *in vivo*. The *in vivo*-generated viral RNA initiates limited replication of the vaccine virus, which in turn leads to efficient immunization. This technology allows the plasmid DNA to launch a live-attenuated vaccine *in vitro* or *in vivo*. Less than 10 ng of pTC83 iDNA encoding the full-length genomic RNA of the TC-83 vaccine strain initiated replication of the vaccine virus *in vitro*. In order to evaluate this approach *in vivo*, BALB/c mice were vaccinated with a single dose of pTC83 iDNA. After vaccination, all mice seroconverted with no adverse reactions. Four weeks after immunization, animals were challenged with the lethal epidemic strain of VEEV. All iDNA-vaccinated mice were protected from fatal disease, while all unvaccinated controls succumbed to infection and died. To our knowledge, this is the first example of launching a clinical live-attenuated vaccine from recombinant plasmid DNA *in vivo*.


## 1. Introduction

DNA vaccines represent a promising technology due to their safety, ease of production, genetic stability, no need for cold chain, and activation of innate immunity pathways [1,2]. However, the clinical application of DNA vaccines has been limited. While DNA vaccines have been recently approved for veterinary applications, low immunogenicity in humans is the major obstacle [3]. In contrast, live-attenuated vaccines are among the most cost-effective and broadly used public health interventions representing approximately 60% of all licensed vaccines and providing long-term immunity following a single-dose vaccination [4]. Live vaccines have own limitations including the need for biocontainment during production, cold chain requirements, and safety concerns due to the possibility of reversion, especially for RNA viruses. These limitations have restricted the use and approval of live-attenuated viral vaccines due to potential adverse events in immunocompromized individuals and the possibility of genetic reversion to the virulent, wild-type phenotype. One example of restricted use of live vaccine is the TC-83 vaccine for Venezuelan equine encephalitis virus (VEEV). VEEV causes epizootics and epidemics in the North, Central, and South America including an outbreak in Texas in 1971 [5,6]. Climate, ecological changes and international travel have increased the risk of VEEV re-emergence [7–9]. The virus can also be easily produced in large quantities and aerosolized as a biological weapon [7,10]. The potentially threatening effects of the VEEV re-emergence demand an effective vaccine [11].

The experimental, live-attenuated TC-83 vaccine [12] is currently the only live vaccine used under an Investigational New Drug (IND) protocol for immunization of medical personnel at risk [7,13,14]. The TC-83 vaccine provides protection against many epizootic viruses of the VEEV complex [15] including IAB, IC, and IE. However, the vaccine can cause adverse effects such as headache and fever in approximately 23% of vaccinees. Another approximately 18% of vaccine recipients do not develop sufficient neutralizing antibody titers [16]. Genetic reversions in the TC-83

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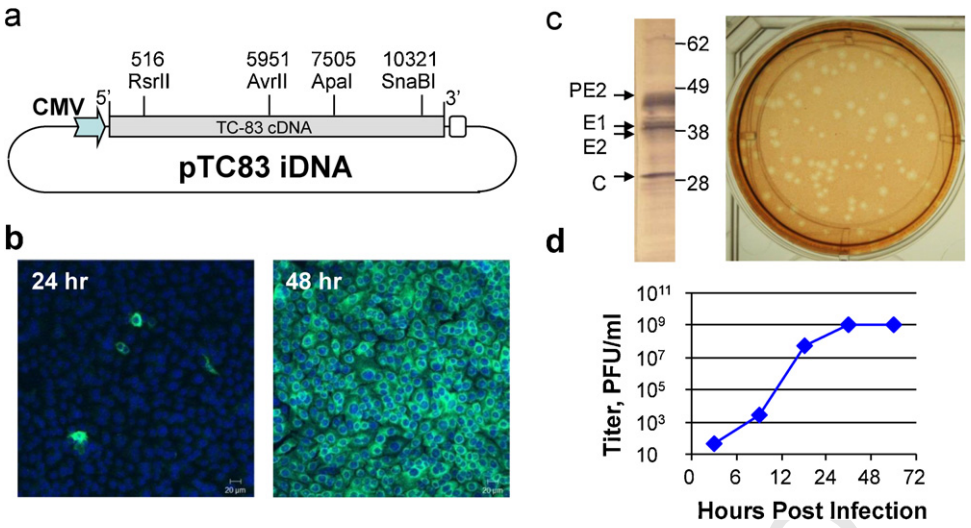


**Fig. 1.** Preparation of pTC83 iDNA containing the full-length TC-83 cloned genome and generation of TC-83 virus in transfected CHO cells. (a) Schematic representation of pTC83 plasmid. Restriction sites used for preparation of the full-length TC-83 clone are indicated. (b) Indirect immunofluorescence assay (IFA) of CHO cells transfected with pTC83 iDNA. IFA was performed at 24 h (left panel) and 48 h post electroporation. In order to visualize nuclei in transfected cells, the 4′,6-diamidino-2-phenylindole (DAPI) stain was used. (c) Western blot of CHO cells transfected with pTC83 iDNA (left panel) and plaque assay of the supernatant from CHO cells transfected with pTC83 iDNA (right panel). Western blot was performed at 24 h post electroporation using ATCC antiserum against VEEV. Plaque assay was carried out in Vero cell monolayers. (d) Replication of iDNA-derived TC-83 virus in infected Vero cells. Vero cells were infected with 100 PFU of iDNA-derived TC-83 virus. Plaque titer was determined in duplicates, error bars are not visible at the log scale shown.

virus have been associated with adverse effects [17]. RNA viruses have high rates of mutations [18,19], which contribute to genetic instability and accumulation of potentially harmful mutations during virus passages for vaccine production.

Due to its long record of clinical use, TC-83 represents a logical starting point for preparation of a safer and better vaccine against VEEV [17]. Here, we describe a novel immunization DNA (iDNA) vaccine platform that potentially can overcome the weaknesses of the TC-83 vaccine by combining the advantages of DNA immunization with the efficacy of live-attenuated vaccine. The pTC83 iDNA vaccine represents a recombinant plasmid that encodes the entire genomic RNA of TC-83 virus under control of eukaryotic promoter. Upon vaccination, iDNA plasmid drives transcription of viral RNA *in vivo* and initiates limited replication of a genetically defined, TC-83-like vaccine virus. Thus, a live attenuated vaccine is launched from iDNA *in vivo*, with no need for external cell substrates or virus passages for vaccine production, which minimizes the potential for reversions or adverse effects, ensures genetic stabilization, and results in efficient immunization. Thus, iDNA vaccine technology allows effective conversion of DNA immunization into a highly immunogenic live attenuated vaccine and combines advantages of both vaccine platforms.

## 2. Materials and methods

### 2.1. Cells and viruses

Baby hamster kidney (BHK-21), Chinese hamster ovary (CHO) and Vero cell lines were obtained from the American Type Culture Collection (Manassas, VA) and maintained in a humidified incubator at 37 °C in 5% $CO_2$ in αMEM supplemented with 10% fetal bovine serum (FBS) and gentamicin sulfate (10 μg/ml) (Life Technologies, Carlsbad, CA). The TC-83 live-attenuated vaccine was obtained from the U.S. Army Medical Research and Materiel Command (Fort Detrick, MD), amplified once in CHO cells and stored at -80°C. The Trinidad donkey strain of VEEV, a 1943 subtype IAB isolate from an epidemic/epizootic [20], is a standard challenge stock and was used at the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID, Fort Detrick, MD). VEEV strain 3908 virus, a 1995 epidemic subtype IC isolate (Weaver et al., 1996), is a standard challenge stock that was used at the University of Texas Medical Branch (UTMB, Galveston, TX).

### 2.2. Plasmids and iDNA preparation

TC-83 vaccine virus was propagated in CHO cells in 75 $cm^2$ flask. At 48 h post infection, the virus was harvested, clarified, and frozen at −80 °C in 1 ml aliquots. Viral RNA was extracted by Trizol LS (Life Technologies). Four cDNA fragments were generated by using One-Step RT-PCR system with specific oligonucleotide primers. Then, cDNA fragments were assembled within pcDNA3.1-derived plasmid under control of CMV promoter.

### 2.3. Transfections and in vitro assays

CHO and Vero cells were transfected by electroporation of plasmid iDNA at concentrations ranging from 8 ng to 5 μg in 75 $cm^2$ flasks. Transfection of CHO and Vero cells was done essentially as described previously [21]. As controls, cells were infected with $10^2$–$10^5$ PFU of TC-83 virus. For virus growth curves, virus samples were harvested at indicated intervals and quantitated in duplicates by a standard plaque assay in Vero cells.

### 2.4. Immunizations and challenge

The iDNA plasmid was isolated from *E.coli* by an endotoxin-free method (Qiagen, Valencia, CA) and formulated in phosphate-buffered saline (PBS) to a concentration of 1 mg/ml. Prior to vaccinations, three-week-old female BALB/c mice were anesthetized with isoflurane. Mice were vaccinated intramuscularly (i.m.) with 50 μl of iDNA in the medial thighs, followed by *in vivo* electroporation at an amplitude of 100 V with pulse duration of 50 msec and an interval between pulses of 200 msec. Controls similarly received unrelated pcDNA3.1-based plasmid DNA in PBS. Animals were electroporated at the site of injection using a two-pin electrode and a square wave electroporator (ECM 830, BTX Genetronics, San Diego, CA). Blood samples were collected from the retro-orbital sinus to detect



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