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Can understanding the virulence mechanisms of RNA viruses lead us to a vaccine against eastern equine encephalitis virus and other alphaviruses?

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SUMMARY

Eastern equine encephalitis virus (EEEV) is a highly neurovirulent mosquito-borne alphavirus that causes severe morbidity and mortality upon human infection. Recent emergence of EEEV into non-endemic regions in the US and Panama demonstrates the need for the development of an effective EEEV vaccine for licensure for human use. The current EEEV vaccine is available to only at-risk laboratory workers but is poorly immunogenic and requires multiple boosters. In this editorial, we summarize recent developments in understanding alphavirus virulence mechanisms that could be utilized to rationally design a live attenuated vaccine against EEEV or other alphaviruses.

Keywords

EEEV; alphavirus; live attenuated vaccine; virulence mechanisms; rational design; VEEV

In recent years, the spread of mosquito-borne diseases into previously non-endemic areas (e.g., West Nile flavivirus into the United States (US) and chikungunya (CHIKV) alphavirus into Europe and the Caribbean) has raised concern and increased the urgency for developing effective vaccines to combat these pathogens. Similarly, the known geographical range of EEEV and incidence of disease has increased in the Eastern US and also has recently emerged in Panama [1,2]. While relatively rare in humans, EEEV causes one of the most severe acute viral infections resulting in 30–70% mortality amongst symptomatic cases [3]. Both Venezuelan equine encephalitis alphavirus (VEEV) and EEEV have the capacity to cause severe disease after aerosol delivery, which raises concern for the possibility of their use as agents of biowarfare/ bioterrorism [4].

A formalin-inactivated EEEV vaccine is currently under Investigation New Drug (IND) status and is available to laboratory researchers; however, immunization can be cost-

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prohibitive, and, like many inactivated vaccines, can require multiple boosters to induce a protective immune response [5]. Two EEEV live attenuated vaccines (LAV), a chimeric Sindbis (SINV) virus encoding the structural proteins of EEEV and a similar vaccine encoding an internal ribosome entry site (IRES) in the subgenomic promoter, have shown protective efficacy in animal models but as yet are unlicensed [6,7]. A LAV against VEEV is similarly available as an IND, but is not likely to be approved due to poor immunogenicity and adverse reactions in 20% of vaccines [8]. Finally, a cell culture adapted CHIKV LAV (181/25) has been given to at-risk researchers, but incomplete attenuation has also led to adverse events [9]. The “gold standard” for LAVs is the yellow fever virus (YFV) 17D vaccine that induces life long immunity in humans, but, even with this vaccine, adverse events can occur after vaccination [10]. In the following paragraphs, we summarize recent advancements in understanding the pathogenesis of alphaviruses and especially, EEEV, which we feel can be utilized to design next-generation LAVs.

The VEEV LAV, TC-83, encodes two attenuating point mutations that i) increase replication of the genomic RNA in relation to production of subgenomic RNA while also altering the stem-loop conformation of the 5' non-translated region (NTR) of the viral RNA genome rendering TC-83 sensitive to IFIT1-mediated antiviral restriction, or ii) altering binding to the host glycosaminoglycan heparan sulfate (HS) [11,12]. Importantly, wild-type VEEV strains evade binding of IFIT1 through changes in the stem-loop conformation of the 5' NTR thus evading one aspect of the host antiviral system ([12]. Incorporating mutations to render viral RNA sensitive to IFIT1 antiviral restriction will be an important step in the generation of an alphavirus LAV.

The acquisition of a positively charged amino acid in the E2 glycoprotein of TC-83 renders the virus an efficient binder of HS structures [13]. Passage in culture, natural variation or deliberate mutagenesis can alter HS binding versus wild type alphavirus strains, typically increasing it with poorly binding viruses (e.g., CHIKV and VEEV), while lowering or altering its qualities with EEEV, thereby, attenuating the virus in animal models of disease with attenuation mechanisms different between the viruses [13–16]. Adaptation to HS binding has been proposed as a rational method for development of alphavirus LAVs previously and should be considered for next-generation LAVs [14,16].

During virus replication, alphavirus proteins arrest host macromolecular synthesis by inhibiting host cell translation and transcription, potentially antagonizing innate immune responses to infection [17]. Mutations in the capsid protein render EEEV or VEEV unable to shut-off host gene transcription thereby attenuating the virus *in vivo* [18,19]. Further, mutations in nsP2 have been identified that affect macromolecular synthesis shutoff and IFN signaling inhibition [20,21]. However, the nature of the mutations and their effects are different for each alphavirus. The addition of mutations to a LAV for EEEV or other alphaviruses that remove/attenuate their ability to shut off host macromolecular synthesis or IFN signaling inhibition will further limit the ability of EEEV to replicate and potentially progress to encephalitic disease.

EEEV also encodes a virulence mechanism in the 3' NTR that contributes to the limited prodrome seen during infection prior to manifestations of encephalitic disease [22,23].

Remarkably, we found that the 3' non-translated region (NTR) of EEEV RNA genome encodes binding sites for the myeloid-specific microRNA (miRNA), miR-142-3p. Binding of the miRNA to EEEV represses viral translation and replication only in myeloid cells likely limiting both innate and acquired immune responses to EEEV. Removal of the microRNA binding sites rescued myeloid cell replication both *in vitro* myeloid cell lines and *in vivo* in myeloid cells in the popliteal lymph node. Importantly, this myeloid cell replication *in vivo* increased production of systemic type I IFN and attenuated the mutant viruses demonstrating a correlation between type I IFN production and the attenuation of EEEV [23]. In combination with removal of the miR-142-3p binding sites, alteration of HS binding of EEEV increased replication in lymphoid tissues, type I IFN production, and prodrome in comparison to viral mutants eliminating only miR-142-3p binding or HS binding [23]. Therefore, an effective EEEV LAV would need both HS and miR-142-3p binding altered to efficiently replicate in peripheral lymphoid tissues inducing a protective immune response. Currently, it is unclear whether or not other alphaviruses utilize miRNA inhibition to determine tissue tropism *in vivo*.

In addition, even though humans are considered dead-end hosts for EEEV, an ideal EEEV LAV candidate would not be replication competent in mosquitoes, preventing transmission from vaccinees to mosquitoes as was seen for the LAV VEEV during an equine vaccination campaign during an epizootic outbreak [24]. Interestingly, removal of the miR-142-3p binding sites from EEEV and the EEEV IRES LAV were unable to establish viral replication in mosquitoes thus eliminating this potential transmission from vaccinees to mosquitoes [6,23].

Both the CHIKV 181/25 and VEEV TC-83 LAV induce adverse events in vaccinees suggesting that the attenuating mutations are not stable and improvements are needed before mass vaccinations [8,9]. To generate a LAV for EEEV, this risk of reverting to virulent phenotypes will need to be considered and mitigated. For example, rather than mutating a single nucleotide, multiple changes in the 5' NTR nucleotide sequence may be needed to stably change the stem-loop structure or multiple mutations altering HS binding could be combined rather than a single mutation as commonly found in LAVs derived from blind passage [16]. This will be particularly important with viruses such as EEEV that cause extremely severe disease at high frequency.

We propose the formal testing of the individual and combined effects of 5' NTR, nsP2, E2 glycoprotein and, with EEEV, 3' NTR mutations upon attenuation and reversion potential as a candidate strategy for an improved alphavirus LAV. The recent discoveries of the mechanisms of action of virulence factors contributing to alphavirus pathogenesis suggest that these can be utilized to rationally design a LAV for EEEV or other arboviruses. However, as noted above, mutations can have different consequences for the different viruses or some may only be applicable to a subset (e.g., miRNA restriction of EEEV) such that thorough empirical testing is required for each combination and virus. The current vaccines are either weak, inactivated formulations or can induce adverse events in vaccinees that will not be suitable for mass vaccinations in the case of widespread outbreak or malicious release. We believe that a novel LAV vaccine against EEEV can be developed to

induce a protective immune response while limiting virulence and potential reversions to virulence, and that this strategy will be broadly applicable to other arboviruses.

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