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Transmission Potential of Two Chimeric Chikungunya Vaccine Candidates in the Urban Mosquito Vectors, *Aedes aegypti* and *Ae. albopictus*

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Abstract. Chikungunya virus (CHIKV) is an emerging, mosquito-borne alphavirus that has caused major epidemics in Africa and Asia. We developed chimeric vaccine candidates using the non-structural protein genes of either Venezuelan equine encephalitis virus (VEEV) attenuated vaccine strain TC-83 or a naturally attenuated strain of eastern equine encephalitis virus (EEEV) and the structural genes of CHIKV. Because the transmission of genetically modified live vaccine strains is undesirable because of the potentially unpredictable evolution of these viruses as well as the potential for reversion, we evaluated the ability of these vaccines to infect the urban CHIKV vectors, *Aedes aegypti* and *Ae. albopictus*. Both vaccine candidates exhibited significantly lower infection and dissemination rates compared with the parent alphaviruses. Intrathoracic inoculations indicated that reduced infectivity was mediated by midgut infection barriers in both species. These results indicate a low potential for transmission of these vaccine strains in the event that a vaccinee became viremic.

INTRODUCTION

Chikungunya virus (CHIKV; *Togaviridae*: *Alphavirus*) is a mosquito-borne pathogen transmitted primarily by *Aedes* (Stegomyia) mosquitoes. The word chikungunya literally means that which bends up in the Makonde language, describing the typical posture of infected individuals when experiencing severe, often chronic joint pain.[1] CHIKV was first isolated in Tanzania in 1953[1] and has since caused sporadic epidemics throughout Africa, India, and Southeast Asia, affecting hundreds of thousands of people.[2,3] Clinical manifestations associated with CHIKV infection include fever, rash, and often incapacitating, severe joint pain.[1,4] Fatalities are historically considered rare and are typically limited to pediatric patients presenting with a hemorrhagic disease followed by shock.[5,6] CHIKV has recently been added to the National Institute of Allergy and Infectious Disease (NAID) priority pathogen biodefense list as a Category C pathogen (http://www3.niaid.nih.gov/topics/BiodefenseRelated/Biodefense/research).

During past epidemics, CHIKV typically circulated in an *Ae. aegypti*-borne human–mosquito–human transmission cycle.[7,8] However, the current epidemic that began in La Reunion and other islands in the Indian Ocean in 2005 and continues in this region as well as in Asia involved a new vector species, *Ae. albopictus* (Skuse).[9] Since the 1980s, this species has become abundant and widely distributed in urban areas of Africa, Europe, and the Americas as well as in its native distribution of Asia.[10] Along with the long-established *Ae. aegypti* populations in tropical and subtropical regions of the world, *Ae. albopictus* has the potential to widen the endemic/epidemic range of CHIKV, raising grave concerns for immunologically naive populations.[11]

Currently, there is no licensed vaccine for CHIK, despite its importance as an emerging, debilitating disease and a potential biological weapon. The only CHIK vaccine to be tested in humans, the 181/clone 25 strain derived by plaque to plaque MRC-5 cell culture passage of a wild-type Thai strain,[12] is attenuated and immunogenic in nonhuman primates and mice and highly immunogenic in humans. However, a small percentage of human vaccinees developed arthritic symptoms during phase II safety studies.[13]

To overcome the reactogenicity and reversion potential of the 181/clone 25 vaccine, we developed chimeric vaccine candidate constructs with the genetic backbone and non-structural protein genes derived from either Venezuelan equine encephalitis virus (VEEV) vaccine strain TC-83 or the naturally attenuated South American eastern equine encephalitis virus (EEEV) strain Br 436087 and the structural protein genes from the La Reunion strain of CHIKV. Both of these vaccine candidates replicate efficiently in cell culture, are highly attenuated in mice, and protect from lethal CHIKV challenge.[14]

Live attenuated vaccines offer many advantages over their inactivated counterparts, including rapid and relatively long-lived immunity after a single immunization. However, live attenuated vaccines also pose the risk for natural transmission if appropriate shedding or viremia occurs. This risk is of particular concern for genetically modified viruses, which might evolve unpredictably during natural circulation. Although our chimeric CHIKV vaccines minimize this risk by producing little or no viremia in animal models,[14] we evaluated their ability to infect natural CHIKV vectors to assess their overall environmental safety. We exposed the two primary epidemic vectors, *Ae. aegypti* and *Ae. albopictus*, to high titer artificial blood meals to evaluate the ability of these viruses to infect, disseminate, and be transmitted.

MATERIALS AND METHODS

Viruses. Three parental alphavirus strains and two chimeric vaccine candidates[14] were evaluated. The parental strains included VEEV vaccine strain TC-83, a naturally attenuated South American strain of EEEV, BeAn436087,[15] and the CHIKV La Reunion (LR) strain, which was isolated from a human during the 2006 La Reunion outbreak and produced from a cDNA clone.[16] The first chimeric vaccine candidate (VEE/CHIKV) consists of the genetic backbone and non-structural protein genes from VEEV vaccine strain TC-83 and the structural protein genes from the CHIKV LR strain. The second candidate (EEE/CHIKV) also contains the structural protein genes from CHIKV LR; however, the backbone and

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non-structural protein genes were derived from the naturally attenuated South American EEEV strain BeAn436087. *In vitro* transcription, transfection, and production of chimeric viruses were performed as described previously.[16]

Mosquito infections. *Ae. aegypti aegypti* sensu stricto and *Ae. albopictus* colonies originating from adult collections made in Galveston, TX (29°13.13′ N, 94°56.06′ W) were allowed to ingest artificial blood meals containing each chimera (VEE/CHIKV or EEE/CHIKV) or one of the parental viruses (TC-83, EEEV, or CHIKV). Cohorts of 50 adult females, 6–10 days post-emergence, were placed in 0.9-L cartons and sucrose-starved for 24 hours before being allowed to feed on artificial blood meals. The blood meals contained 33% (v/v) defibrinated sheep erythrocytes (Colorado Serum Company, Denver, CO), 33% (v/v) heat-inactivated fetal bovine serum (Omega Scientific Inc., Tarzana, CA), 33% (v/v) of each virus in cell culture medium, with a final concentration of approximately 6 $\log_{10}$ PFU/mL, and 1% (v/v) 0.25 μM adenosine triphosphate. Artificial blood meals were contained within collagen membranes to simulate animal skin and were warmed to 37°C using a Hemotek feeder (Discovery Workshops, Accrinton, UK) before being placed on the screened lids of 0.9-L cartons. Mosquitoes were allowed to feed for ~1 hour, and were then cold-anesthetized on ice for sorting; after this process, engorged females were incubated at 27°C with a relative humidity of ~70% for a 10-day extrinsic incubation period. For each experiment, mosquitoes that engorged on blood meals with no virus served as negative controls, and none became infected.

Intrathoracic mosquito inoculations. Cohorts of 10–20 adult *Ae. aegypti* and *Ae. albopictus* females were injected intrathoracically with 1–2 μL of a 6 $\log_{10}$ PFU/mL stock of each chimeric virus. Mosquitoes were held for an extrinsic incubation period of 10 days at 27°C at a relative humidity of 70% before being processed.

Mosquito processing. After extrinsic incubation, mosquitoes were cold-anesthetized, and the legs and wings were removed. The proboscis was inserted into a 10-μL capillary tube containing immersion oil (Cargille Laboratories, Cedar Grove, NJ) to induce salivation for 1 hour. Each saliva sample was transferred into a separate tube containing 100 μL 10% fetal bovine serum in minimal essential medium (FBSMEM). Bodies and legs/wings were put in individual tubes containing 350 μL 10% FBS/MEM and a stainless steel bead for trituration. Intrathoracically inoculated mosquitoes were placed in tubes without dismemberment or saliva collection and stored at −80°C. Each body and leg/wing sample was triturated for 4 minutes using a Mixer Mill 300 (Retsch, Newton, PA). Samples were then centrifuged, and individual sample supernatant was then inoculated onto African green monkey Vero cells and observed for cytopathic effects (CPE) for 7 days. Cytopathic effect observed in Vero cell cultures was used as an indicator for the presence of virus.

Statistical analyses. Infection, dissemination into the hemocoel, and saliva infection rates were compared among virus groups using a χ^2 for independence test with InStat 3.0 for Macintosh (Graph-Pad Software, San Diego, CA). Results were considered significant if $P \leq 0.05$.

RESULTS

***Ae. aegypti*.** After exposure to high titer blood meals, *Ae. aegypti* body infection rates were consistently high for the parental viruses (TC-83, CHIKV, and EEEV: 100%, 100%, and 95%, respectively) (Table 1). Both TC-83/CHIKV and EEE/CHIKV vaccine strains showed significantly lower infection and dissemination rates (0% and 38%, respectively) compared with their respective parental viruses ($P < 0.0001$ and $P < 0.0025$, respectively). To assess whether the chimeric viruses could be transmitted, we performed an additional oral infection experiment and collected saliva from extrinsically incubated mosquitoes. Strain TC-83/CHIKV was not detected in the saliva in any of the mosquitoes tested, whereas EEE/CHIKV resulted in only 1 positive saliva sample of 15 samples taken from exposed mosquitoes (Table 2).

To determine whether the barrier to oral infection exhibited by the chimeric viruses was related to initial infection of the midgut, intrathoracic inoculations were performed. The bodies of all 10 mosquitoes inoculated with TC-83/CHIKV or EEE/CHIKV were infected after 10 days of incubation, indicating that a midgut infection barrier limited oral infection by the chimeric strains.

***Ae. albopictus*.** *Ae. albopictus* was also highly susceptible to oral infection and dissemination for all parent viruses (≥ 90%) (Table 3). Overall infection rates were similar to those observed in infected *Ae. aegypti* for both parental viruses and the vaccine candidates. Both TC-83/CHIKV and EEE/CHIKV showed a decreased infection and dissemination compared with their respective parent viruses ($P < 0.0001$). Similarly to *Ae. aegypti*, the ability of the chimeras to infect the saliva of *Ae. albopictus* was severely diminished, with only 1 positive of 15 exposed to EEE/CHIKV and 0 positive of 20 exposed in the TC-83/CHIKV group (Table 4), suggesting a low transmission potential. To determine if *Ae. albopictus* exhibited a midgut infection barrier for TC-83/CHIKV and EEE/CHIKV, we performed intrathoracic inoculations. Both chimeras were found to be capable of efficient (100%; $N = 9$ and $N = 13$, respectively) intrathoracic infection, indicating the presence of a midgut oral infection barrier in *Ae. albopictus*.

DISCUSSION

Currently, there is no licensed vaccine for CHIK, despite its recent emergence on a global scale. A live attenuated vaccine candidate developed during the 1980s proved to be reactogenic in humans[13] and was also capable of infecting potential mosquito vectors.[17] Recently, a chimeric approach to developing a safer CHIK vaccine resulted in two promising candidates.[14] Therefore, the purpose of our study was to assess whether these chimeric CHIK vaccine candidates have the potential to enter into a human–mosquito–human transmission cycle in the event that a person becomes viremic after vaccination.

Table 1
Infection and dissemination of EEE/CHIKV and TC-83/CHIKV vaccine strains and parent viruses in *Ae. aegypti* mosquitoes

Virus strain*	Blood meal titer ($\log_{10}$ PFU/mL)	Number engorged	No. infected (%)†	
			Body	Legs/wings (disseminated)
EEE/CHIKV	7.1	21	8 (38)	8 (38)
TC-83/CHIKV	7.3	26	0 (0)	0 (0)
TC-83	7.4	24	24 (100)	23 (96)
CHIK-LR	7.7	27	27 (100)	26 (96)
EEEV	6.4	19	18 (95)	17 (90)

* EEEV strain BeAn436087; CHIKV strain La Reunion.
† Legs/wings were only tested from mosquitoes with bodies infected.

Table 2
Infection, dissemination, and transmission potential of chimeric CHIK vaccine strains in *Ae. aegypti* mosquitoes

Virus strain*	Blood meal titer (log_{10} PFU/mL)	Number engorged	No. infected (%)†		
			Body	Legs/wings	Saliva
EEE/CHIK	7.4	15	5 (33)	4 (27)	1 (7)
TC-83/CHIK	7.2	20	3 (15)	1 (5)	0 (0)

* EEEV strain BeAn436087; CHIKV strain La Reunion.
† Legs/wings and saliva were only tested from mosquitoes with bodies infected.

Table 4
Infection, dissemination, and transmission potential of chimeric CHIK vaccine strains in *Ae. albopictus* mosquitoes

Virus strain*	Blood meal titer (log_{10} PFU/mL)	Number engorged	No. infected (%)†		
			Body	Legs/wings	Saliva
EEE/CHIK	7.4	15	5 (33)	4 (27)	1 (7)
TC-83/CHIK	7.2	20	3 (15)	1 (5)	0 (0)

* EEEV strain BeAn436087 and CHIKV strain La Reunion.
† Legs/wings and saliva were only tested from mosquitoes with bodies infected.

Using the two principal urban CHIKV vectors, *Ae. aegypti* and *Ae. albopictus*,[11] we assessed the potential of each vaccine (TC-83/CHIKV and EEE/CHIKV) to infect, disseminate, and be shed into the saliva compared with the parental viruses (VEEV vaccine strain TC-83, CHIKV, and EEEV).

The parental CHIKV LR strain, VEEV vaccine strain TC-83, and the EEEV (BeAn436087) strain were all highly infectious for *Ae. aegypti* and *Ae. albopictus* when large oral doses were provided. However, the TC-83/CHIKV vaccine strain was virtually incapable of oral infection and dissemination into the hemocoel of either mosquito species. The EEE/CHIKV strain showed slightly more efficient infection and dissemination in *Ae. aegypti* and *Ae. albopictus*, although at significantly lower levels than the parental viruses. This was surprising considering that all three parental viruses were similarly highly infectious for both mosquito species. This could reflect greater compatibility between the CHIKV-derived structural protein genes and the other genome regions found in the EEEV strain.

To assess whether TC-83/CHIKV and EEE/CHIKV were capable of replicating within *Ae. aegypti* and *Ae. albopictus* after entry into the hemocoel, we performed intrathoracic inoculations, bypassing the midgut infection. In contrast to oral exposure, the intrathoracic route infected all mosquitoes tested, suggesting that the vaccine strains are capable of sustained replication in these vectors after the midgut infection barrier is circumvented.

Similar mosquito transmission studies have been performed recently with other chimeric alphaviruses. Chimeric EEEV vaccine candidates with the nonstructural protein genes from Sindbis virus (SINV) and the structural protein genes from either a North or South American EEEV were unable to infect and disseminate when high titer blood meals (up to 7.4 log_{10} PFU) were provided to *Ae. sollicitans* (Walker) or *Ae. taeniorhynchus* (Wiedemann).[18] Similarly, an oral dose of 6.1–6.6 log_{10} PFU of a chimeric SINV/Western equine encephalitis virus (SIN/WEEV) vaccine candidate was generally inefficient at infecting the primary WEEV vector, *Culex tarsalis* (Coquillett), compared with the parental viruses.[19] In addition, the ChimeriVax family of flavivirus vaccine candidates, ChimeriVax^{T}-JE, ChimeriVax^{T}-DEN1-4, and YF-VAX (17D), all show reduced infectivity and transmission potential in their respective vectors when given in large oral doses,[20–22] further validating the ecological safety of the chimeric vaccine design strategy.

Table 3
Infection and dissemination of EEE/CHIKV and TC-83/CHIKV vaccine strains and parent viruses in *Ae. albopictus* mosquitoes

Virus strain*	Blood meal titer (log_{10} PFU/mL)	Number engorged	No. infected (%)†	
			Body	Legs/wings (disseminated)
EEE/CHIKV	7.1	27	7 (26)	6 (22)
TC-83/CHIKV	7.3	28	1 (3)	0 (0)
TC-83	7.4	30	30 (100)	29 (96)
CHIK-LR	7.7	24	24 (100)	24 (100)
EEEV	6.4	20	20 (100)	19 (95)

* EEEV strain BeAn436087; CHIKV strain La Reunion.
† Legs/wings were only tested from mosquitoes with bodies infected.

The yellow fever 17D vaccine strain backbone is known to be poorly infectious for *Ae. aegypti*,[23] probably explaining the poor infectivity of the ChimeriVax vaccines. However, the genetic mechanism for the poor infectivity of the chimeric alphavirus vaccine candidates remains unknown. These chimeric alphaviruses replicate efficiently in vertebrate and mosquito cells *in vitro* but invariably exhibit attenuated phenotypes *in vivo*. Suboptimal interactions between the nonstructural and structural proteins or between the structural proteins or their genes and cis-acting viral RNA sequences may provide a stronger attenuation phenotype *in vivo*, where a diverse set of cells and tissues must be infected for efficient replication and transmission.

In summary, both TC-83/CHIKV and EEE/CHIKV vaccine candidates are poorly infectious for the primary urban CHIKV vectors *Ae. aegypti* and *Ae. albopictus*. TC-83/CHIKV is completely unable to infect either species after high titer oral doses, suggesting slightly better environmental safety. However, we believe both vaccine candidates are sufficiently attenuated for mosquito infection to warrant their further development as human vaccine candidates for the prevention of CHIKV in humans.

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