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Cross-Protective Immunity against O`Nyong-nyong Virus Afforded by a Novel Recombinant Chikungunya Vaccine

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Abstract

Emerging mosquito-borne alphavirus infections caused by chikungunya virus (CHIKV) or o`nyong-nyong virus (ONNV) are responsible for sporadic and sometimes explosive urban outbreaks. Currently, there is no licensed vaccine against either virus. We have developed a highly attenuated recombinant CHIKV candidate vaccine (CHIKV/IRES) that in preclinical studies was demonstrated to be safe, immunogenic and efficacious. In this study we investigated the potential of this vaccine to induce cross-protective immunity against the antigenically related ONNV. Our studies demonstrated that a single dose of CHIKV/IRES elicited a strong cross-neutralizing antibody response and conferred protection against ONNV challenge in the A129 mouse model. Moreover, CHIKV/IRES immune A129 dams transferred antibodies to their offspring that were protective, and passively transferred anti-CHIKV/IRES immune serum protected AG129 mice, independently of a functional IFN response. These findings highlight the potential of the CHIKV/IRES vaccine to protect humans against not only CHIKV but also against ONNV-induced disease.

Keywords

Chikungunya virus (CHIKV); O`Nyong –nyong virus (ONNV); A129 mice; Interferon (IFN); maternal-fetal immunity

1. Introduction

Emerging mosquito-borne alphavirus infections caused by chikungunya virus (CHIKV) or o`nyong-nyong virus (ONNV) are responsible for sporadic and sometimes explosive epidemics characterized by debilitating pain and inflammation of musculoskeletal tissue. In

particular, ONNV which is transmitted by *Anopheles* mosquitoes, was the cause of a major epidemic of debilitating joint pain that began in Uganda and spread over Eastern Africa, affecting at least two million people between 1959 to 1962 [1]. More recently, ONNV was the cause of another epidemic in southern Uganda [2]. A vertebrate reservoir has yet to be identified for this virus. CHIKV has for decades been an important etiologic agent of human disease in Africa and Asia. The virus recently reemerged into the Indian Ocean islands, India and Southeast Asia to cause several million cases of severe and often chronic arthralgia [3, 4]. In addition, reports of autochthonous transmission in southern France [5], and Italy [6] highlight the potential of this virus for worldwide dissemination and impact on public health. Unlike ONNV, CHIKV in Africa appears to be maintained in a sylvatic cycle that involves non-human primates and numerous species of *Aedes* mosquitoes [3, 4]. In Asia the virus circulates between *A. aegypti* and *A. albopictus* mosquitoes and naïve human hosts [3, 4].

Both ONNV and CHIKV belong to the family *Togaviridae*, genus *Alphavirus*. They are enveloped viruses with single-stranded, positive-sense RNA genomes and are grouped under the Semliki Forest Virus antigenic complex on the basis of serological cross-reactivity of their envelope proteins [7]. Typically, infection by these viruses is manifested by rash, headache, fever, viremia (which is short-lived), arthritis, myositis, arthralgia and myalgia. Serological tests have shown strong cross-reactivity of anti-CHIKV antibodies with ONNV whereas the reciprocal cross-reaction is weaker [8, 9]. Despite the close antigenic relationship between CHIKV and ONNV, genetic studies and phylogenetic analyses have clearly demonstrated that these two viruses are genetically distinct [2, 4].

Because humans appear to be the only amplification host during epidemic transmission of CHIKV and ONNV, the best means of controlling their spread is by vaccination. Currently, there is no licensed vaccine available for either. Several attempts to develop a vaccine against CHIKV have been described, including alphavirus chimeras [10], live attenuated virus [11], formalin-killed vaccines [12, 13], consensus-based DNA vaccines [14], a virus-like particle vaccine [15], and an adenovirus vectored vaccine [16]. We recently developed a candidate CHIKV vaccine by employing an attenuation mechanism that also prevents the infection of potential mosquito vectors [17]. The internal ribosome entry site (IRES) from encephalomyocarditis virus was used to replace the sub genomic promoter in a cDNA CHIKV clone, thus altering the level and host-specific mechanism of structural protein gene expression [18]. The testing of the vaccine in both normal outbred mice and interferon response-defective mice demonstrated; i) that it is highly attenuated, immunogenic and efficacious after a single dose, and ii) is incapable of replicating in mosquito cells or infecting mosquitoes *in vivo*, an important safety feature for use in non-endemic locations [18].

In this study we sought to investigate further the capacity of CHIKV/IRES vaccine to cross-protect against the antigenically related ONNV. Our results demonstrated that the vaccine elicited strong cross-neutralizing antibodies against ONNV and conferred protection against challenge with this virus after a single administration. Moreover, the protective role of antibodies was demonstrated in two models; i) CHIKV/IRES immune A129 dams (which lack functional interferon type I receptor; IFN- α/β R^{-/-}) transferred antibodies to their offspring that protected against ONNV challenge, and ii) anti-CHIKV/IRES antibodies conferred protection against ONNV challenge of AG129 mice (which lack functional interferon type I and type II receptors; IFN- α/β - γ) independently of a functional IFN response.

2. Materials and Methods

2.1. Viruses

The virus strains used for plaque reduction neutralization assays were the CHIKV/ 181–25 passage Vero3, and ONNV strain SG650 passage Vero3BHK1. Each virus was diluted in 2ml Dulbecco's Minimum Essential Medium -supplemented with 10% FBS, 100U/ml of penicillin, and 100mg/ml of streptomycin (DMEM Complete Media) to yield a multiplicity of infection (MOI) of 0.1 PFU/cell. Flasks (75 cm²) with confluent Vero cells were seeded with the diluted virus, and were incubated at 37°C for 1 hour with rocking every 20 minutes. At the end of the incubation, cells were rinsed three times with Dulbecco's PBS, and 25 ml of DMEM complete media was added. The flasks were incubated at 37°C until they reached 50% cytopathic effect (CPE) at which point the supernatant was harvested. The harvested supernatant was diluted in 50% FBS and divided into 200µl aliquots for storage at –70°C. Virus titers were determined by plaque assay.

The development of CHIK live-attenuated vaccine was based on a novel attenuation strategy [17] relying on the inactivation of the subgenomic promoter, and the addition of an encephalomyocarditis virus (EMCV) internal ribosome entry sequence (IRES) to drive translation of the structural protein genes [18]. The resulting virus had both a highly attenuated profile and was incapable of infecting mosquitos [18]. An infectious cDNA clone generated from the wild-type (wt) La Reunion strain (CHIKV-LR) was used as the genetic backbone. A detailed description of the construction and characterization of the CHIKV/ IRES candidate vaccine was previously reported by Plante et al [18].

2.2. Virus titer determination

A TaqMan real-time PCR assay was performed to estimate virus concentration [plaque forming equivalents (PFU) per ml] from serum samples. First, viral RNA was isolated from serum using the QiaAmp Viral RNA protocol (Qiagen, Valencia, CA). Total RNA was extracted from 50µl of sample and eluted from the kit columns into a final volume of 60µl of elution buffer. The RNA was stored at –80° C until use. The CHIKV primer set CHIK 243+/CHIK 330- and CHIK 273 probe was designed with the primer select program and were based on the available full-length sequences. The real-time probes were labeled with 5' end FAM reporter dye and 3' end BHQ1 quencher dye. The QuantiTect probe RT-PCR kit (Qiagen, Valencia, CA) was used for the real-time (TaqMan) assay. A standard determined by serially diluting an ONNV virus stock for PFU conversion, titrating each dilution, and then extracting the RNA according to the RNeasy protocol, was used to extrapolate results to virus titers. A curve correlation coefficient of 0.950 and a 90 –100% PCR efficiency was used to validate each assay.

2.3. Plaque reduction neutralization test

Plaque reduction neutralization tests (PRNT) were used to detect neutralizing antibodies in serum samples. Briefly, the samples were heat-inactivated for 30min at 56°C and then serially diluted two-fold in Dulbecco's Minimum Essential Medium supplemented with 10% FBS, 100U/ml of penicillin, and 100mg/ml of streptomycin (Gibco, Carlsbad, CA). A suspension of 100 PFU /125µl was then mixed with the diluted serum samples and the suspension incubated for 1h at 37°C. The serum-virus suspension was then transferred onto 6-well cell culture plates (Corning, Corning, NY) containing a confluent monolayer of Vero cells. The virus/cell mixture was incubated for 1h at 37°C with the plate rocked every 20 min. Each well was then overlaid with a 0.4% Genepure LE agarose/DMEM medium layer (ISC BioExpress, Kaysville, UT) and plates were incubated at 37°C for 48 hours for CHIKV and 72 hours for ONNV. The agarose layer was removed and the wells were covered with a fixative/staining solution (40% methanol and 0.25% crystal violet) for ten minutes. Plates

then rinsed with water to remove the fixative/staining solution. Plaques were counted and titers were calculated and expressed as the reciprocal of serum dilution yielding a >80% reduction (PRNT₈₀) in the number of plaques.

2.4. Animal experiments

2.4.1. Ethics statement—129s/v mice with null mutations in the IFN- α/β receptor (A129) [19] or both IFN- α/β and - γ receptors (AG129) were bred and housed at the University of Wisconsin animal facility (Charmany Farms) under specific pathogen-free conditions. All procedures were carried out in accordance with institutional and NIH guidelines for animal care and use.

2.4.2. Active immunization and passive transfer of maternal antibodies studies in A129 mice-ONNV challenge—Three weeks after a single intradermal (ID) injection of 10⁴ PFU of CHIKV/IRES vaccine or PBS at the footpad of 6–8 week old A129 mice (n=10), immune and non-immune female mice were mated with A129 male mice. Litters were born on day 19, 20 and 21. The pups were allowed to nurse for three weeks before being weaned and challenged. Prior to ID (footpad) challenge with 10⁴ PFU ONNV (in a 50 μ l volume), immune (n=5) and non-immune (n=10) three week-old pups were bled to measure levels of circulating neutralizing antibodies. In all studies phosphate buffered saline (PBS) was used as virus diluent. Similarly, CHIKV/IRES immune and PBS immunized dams were bled to measure neutralizing antibody responses 37 and 65 days post-primary immunization and then challenged with the same dose of ONNV as described above. All mice were monitored for morbidity and mortality over a period of two weeks. In addition, on day 3 post-challenge adult A129 mice were bled to measure viremia. In a separate study two groups of 6–7 week old A129 mice (n=6) were injected (as described above) with either 10⁴ PFU CHIKV/IRES vaccine or PBS (in a 50 μ l volume). Five weeks post-priming mice were bled to measure seroconversion and then challenged ID (footpad) with 10⁵ PFU ONNV (in a 50 μ l volume) on day 38. Morbidity and mortality was recorded for a period of two weeks.

2.4.3. Passive immunization of AG129 mice with anti-CHIK/IRES immune serum-ONNV challenge—To examine whether anti-CHIKV/IRES immune serum can exert a protective effect against ONNV independent of a functional IFN response, two groups of 6–8 weeks old AG129 mice were injected intraperitoneally with a pool of CHIKV/IRES immune serum (n=5) or non-immune serum (n=4), each administered in 150 μ l volume. The immune serum was collected from A129 mice five weeks post-priming with 10⁴ PFU of vaccine virus. Twenty four hours post-passive transfer, mice were challenged ID (footpad) with 10⁵ PFU ONNV (in a 50 μ l volume). Following infection all animals were monitored for morbidity and mortality for two weeks.

3. Results

3.1. Immunization with CHIKV/IRES candidate vaccine elicits cross protective immunity in IFN-type-I deficient A129 mice

A129 mice, which are susceptible to alphavirus infection [19], were selected to test the cross-protective efficacy of CHIKV/IRES vaccine against ONNV challenge. Intradermal administration of a single dose of CHIKV/IRES vaccine elicited an antibody response that neutralized the homologous virus and cross-neutralized ONNV (neutralizing titer of pool serum for both viruses was >320) five weeks post-priming. Challenge of CHIKV/IRES immune animals with 10⁴ PFU ONNV produced no detectable viremia or any footpad swelling (Figure 1). In contrast, control non-immune animals exhibited high levels of viremia on day 3 post-challenge ranging from 3.90 to 5.08 Log₁₀ PFU/ml (individual titers;

4.48, 4.30, 5.079, 4.55 and 3.90). In addition, they developed footpad swelling that lasted for the duration of the study (Figure 1). No significant weight loss or mortality was observed in any of the groups (data not shown). However, when a higher dose of ONNV (10^5 pfu) was used to challenge adult CHIKV/IRES immune mice (5 weeks post-priming) we observed weight loss (Figure 2a) and footpad swelling (Figure 2b) in control but not in immune animals. In addition, half of the control mice succumbed to infection by day 6 post-challenge whereas all immune mice were protected (Figure 2c). Prior to challenge, pooled serum from CHIKV/IRES- immunized mice (collected on day 35 post-prime) had a neutralizing titer of 320 and 160 against CHIKV and ONNV, respectively.

3.2. CHIKV/IRES vaccinated A129 dams transfer antibodies to their offsprings that protect against ONNV challenge

The passive transfer immunity from mothers to their offspring is clearly of great importance to their survival. Since the severity of alphavirus infection is critically dependent on age and functionality of type-I IFN signaling [19] we examined the role of CHIKV/IRES maternal-fetal immunity in protection against ONNV challenge in three week old A129 mice. The anti-ONNV cross-neutralizing antibody titers in the serum of three week old pups derived from CHIKV/IRES immune dams (just prior to ONNV challenge) was >160 (160, 320, >320 , >320 and >320 for each individual pup). Following challenge, pups from CHIKV/IRES immune dams showed no signs of morbidity or mortality (Figure 3a, b, c & d). In contrast, pups from PBS immunized dams had ruffled appearance, hunched posture, weight loss and footpad swelling (Figure 3a, b & c). By day 5 post-challenge all control animals had succumbed to infection (Figure 3d). The difference in the initial body weight between the two groups of challenged pups was due to their gender. CHIKV/IRES immune pups were female whereas control pups were male. Due to practical difficulties in reproducing the exact conditions of this experiment this study was performed only once.

3.3. Anti-CHIKV/IRES antibodies confer protection against ONNV independently of a functional IFN response

Antibodies are believed to play a critical role in protection against alphaviral disease [20–22]. Therefore, we sought to determine if those elicited by the CHIKV/IRES vaccine can protect against fatal ONNV virus infection independently of a functional IFN response. As shown in Figure 4, passively transferred anti-CHIKV/IRES immune serum in AG129 mice conferred 100% protection against ONNV challenge. In contrast, control animals that received normal mouse serum succumbed to infection by day 4 post-challenge. Protected mice had no weight loss as compared to the control animals (data not shown). However, they did develop footpad swelling on day 3 post-challenge that was more pronounced by day 9 (Figure 5).

4. Discussion

CHIKV and ONNV are the causes of several widespread epidemics of debilitating arthritis in Africa and Asia. There is still no licensed vaccine available to prevent disease induced by these viruses. To address this public health concern, an IRES-dependent replication of CHIKV strategy was developed that resulted in a highly attenuated profile of the virus and rendered it incapable of replicating in mosquito cells [18]. This candidate CHIKV/IRES vaccine was safe and immunogenic in mice and conferred protection against wt CHIKV challenge after a single administration [18]. We further evaluated this vaccine for its ability to elicit cross-neutralizing antibodies and protect against the antigenically related ONNV. Active immunization of A129 mice with the CHIKV/IRES candidate vaccine elicited neutralizing antibody responses to the homologous virus that also cross neutralized and fully protected immune animals against ONNV challenge. This finding is consistent with

previous studies indicating that CHIKV and ONNV are antigenically related and therefore share antigenic sites that are identical and/or conformationally similar. Indeed the two viruses share 85% homology in their structural proteins [23, 24]. Comparative studies between CHIKV and ONNV have reported one- way antigenic relationship in which polyclonal antibodies to CHIKV strongly neutralize ONNV [8, 9]. Given the fact that the CHIKV/IRES immune serum is polyclonal it is anticipated that the cross- neutralizing B cell epitopes it recognizes are linear [25] and/or conformational in nature. These epitopes could be residing in either of the two surface E1 and E2 glycoproteins which carry the main antigenic determinants of the virus [26, 27].

The A129 mouse model which is deficient in type-I IFN responses is a promising tool to study symptomatic alphavirus infections [19] and evaluate candidate CHIKV vaccines [18, 22]. In contrast to the wt CHIKV infection in adult A129 mice where a dose as low as 10^{-2} pfu can cause morbidity and mortality [18, 19] in this study, a challenge dose of 10^4 pfu ONNV did not result in any mortality. The only sign of morbidity observed was swelling of the injected hind leg, which lasted for the duration of the study. However, this effect appears to be dependent on the challenge virus dose. Infection of adult A129 mice with a 10^3 pfu dose of ONNV did not produce any morbidity or mortality (data not shown) whereas a dose of 10^5 pfu resulted in a significant weight loss, footpad swelling and partial mortality in the PBS immunized control mice as compared to CHIK/IRES immune mice. The differences in pathogenicity between wt CHIKV and ONNV in A129 mice probably reflect differences in virus virulence in combination with host factors. In the particular case of ONNV its replication in the absence of IFN- α/β signaling could be totally or partially controlled by IFN- γ when administered at a dose of 10^3 or 10^4 pfu, respectively. This notion is consistent with previous findings from studies comparing the attenuation profile of CHIKV/IRES and CHIKV 181/25 vaccines in the A129 mouse model [18, 22].

In alphavirus infections, neutralizing antibody responses have been shown to be critical in protection [20–22]. The role of CHIKV/IRES-induced neutralizing antibodies in protection against ONNV was examined under two different experimental conditions of passive transfer. First, we took advantage of the fact that alphavirus infection in neonatal or very young A129 mice is particularly severe, and this severity correlates with higher viral loads and dissemination to the central nervous system [19]. To this end, we examined the role of maternal-fetal immunity in protection against ONNV challenge of 3-week-old A129 mice. Pups from CHIKV/IRES immune dams were fully protected in the presence of cross-neutralizing anti-ONNV antibodies. It is noticeable that in 3-week-old A129 mice infection with 10^4 pfu of ONNV resulted in rapid mortality of non-immune pups. This is in contrast with findings reported in adult mice in this study. These differences in susceptibility may be accounted for by a number of physiological variables, including the relative proportion of tissue fibroblasts, the rate of cell division, and the maturity and effectiveness of the innate immune system [19, 28].

Our second approach was based on evaluating the efficacy of passively transferred anti-CHIKV/IRES immune serum in AG129 mice that have both IFN-type I and II responses compromised and therefore, are more susceptible to alphaviral disease. Passive transfer of anti-CHIKV/IRES immune serum abrogated lethality associated with ONNV, a finding that is consistent with previous observations with the CHIKV/181/25 vaccine [22] and Sindbis virus [29]. However, passively immunized mice with anti-CHIKV/IRES immune serum did develop swelling at the injected hind leg. Considering the 5-fold dilution of the CHIKV/IRES immune serum to an adult mouse of approximately 800 μ l blood volume (peripheral blood) this should correspond to a cross neutralizing titer of 32 in the mouse blood stream. This suggests that in the absence of a functional IFN response higher titers of cross-

neutralizing antibodies might be required to completely eliminate virus replication upon challenge.

Overall, our findings highlight the efficacy of CHIKV/IRES candidate vaccine in preclinical mouse models for active (at least for short term) and passive protection against ONNV challenge. An important aspect of this cross-protective humoral immune response that needs to be addressed is its duration. Future clinical trials with the CHIKV/IRES candidate vaccine would be relevant in answering this question since kinetics of cross-neutralizing antibody responses will be measured in the human host.

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Highlights

The CHIKV/IRES vaccine elicits cross-neutralizing antibody responses against ONNV

CHIKV/IRES immune animals are protected against ONNV challenge

Protection is mediated by antibodies as evident by passive transfer studies

CHIKV/IRES immune dams transfer protective anti-ONNV antibodies to their offspring

Figure 1.

Footpad swelling over time after challenge of adult A129 CHIKV/IRES immune mice with ONNV. Two groups of A129 mice received a single footpad injection of 10^4 pfu CHIKV/IRES vaccine or PBS in $50\mu\text{l}$ volume. Nine weeks post-priming they were challenged by the same route with 10^4 pfu ONNV in $50\mu\text{l}$ volume. Following challenge they were monitored over a period of two weeks. Bars represent average footpad swelling of groups of 5 animals $\pm$ standard deviation.

Figure 2.

Weight loss (a), footpad swelling (b) and survival (c) of adult A129 mice immune to CHIKV/IRES or non-immune controls (injected with PBS) following challenge with 10^5 pfu ONNV. Two groups of A129 mice ($n=6$) received a single footpad injection of 10^4 pfu CHIKV/IRES vaccine or PBS in 50 μ l volume. Thirty eight days post-priming they were

challenged by the same route with 10^5 pfu ONNV in 50 μ l volume. Following challenge they were monitored over a period of two weeks.

Figure 3.

Clinical score (a), weight change (b), footpad swelling (c) and survival (d) of weanling 3-week old A129 mice derived from CHIKV/IRES immune dams (n=5) or PBS immunized dams (n=10) following challenge with 10^4 pfu ONNV. Clinical score (a) was recorded as follows; 0=good health, 1=ruffled, 2=ruffled and hunched, 3=moribound, 4=euthanized/ found dead.

Figure 4. Survival of groups of AG129 mice (n=5) passively immunized via the IP route with anti-CHIKV/IRES immune or normal mouse serum and challenged ID with 10^5 pfu ONNV twenty four hours later. Mice were monitored over a period of two weeks.

(a)

(b)

Figure 5.
A typical picture of footpad swelling in infected AG129 mice. (a) virus injection site (left hind footpad) and, (b) non-injected site (right hind footpad).