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Biotechnological Applications of an Insect-Specific Alphavirus

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The coupling of viral and arthropod host diversity, with evolving methods of virus discovery, has resulted in the identification and classification of a growing number of novel insect-specific viruses (ISVs) that appear to be evolutionarily related to many human pathogens but have either lost or have yet to gain the ability to replicate in vertebrates. The discovery of ISVs has raised many questions as to the origin and evolution of many human pathogenic viruses and points to the role that arthropods may play in this evolutionary process. Furthermore, the use of ISVs to control the transmission of arthropod-borne viruses has been proposed and demonstrated experimentally. Previously, our laboratory reported on the discovery and characterization of Eilat virus (EILV), an insect-specific alphavirus that phylogenetically groups within the mosquito-borne clade of medically relevant alphaviruses, including eastern equine encephalitis virus (EEEV) and Venezuelan equine encephalitis virus (VEEV), as well as chikungunya virus (CHIKV). Despite its evolutionary relationship to these human pathogens, EILV is unable to replicate in vertebrate cells due to blocks at attachment/entry and RNA replication. We recently demonstrated that, using a chimeric virus approach, EILV could be utilized as a platform for vaccine and diagnostic development, serving as a proof-of-concept for other ISVs. Due to the vast abundance of ISVs, there is an untapped resource for the development of vaccines and diagnostics for a variety of human pathogens and further work in this area is warranted.

Keywords: insect-specific virus, Eilat virus, chikungunya, vaccine, diagnostic

Introduction

Historically, research on insect viruses has focused mainly on those related to insects that do important damage to agriculture or on viruses discovered by chance in other insects, sometimes including vectors of human and animal pathogens. This led to a very superficial knowledge of the complete insect virome. Because virus discovery typically follows surveillance implemented in response to disease, knowledge of virus diversity and systematics became highly skewed toward human and agriculturally important pathogens. For example, in the genus *Flavivirus* of the family *Flaviviridae*, major groups of vector-borne animal viruses and animal viruses without known vectors were identified decades ago, but only one insect-specific virus (ISV, capable of infection of insects but not vertebrates) was discovered before the year 2000. Since that time, thanks mainly to next-generation sequencing, dozens of new insect-specific flaviviruses, some with distinct genome organization, have been discovered (Roundy *et al.*, 2017). This has led to major changes in our understanding of the evolution of several virus genera that include arthropod-borne viruses (arboviruses), from the most parsimonious explanation that ISVs evolved from arboviruses along with the loss of vertebrate infection potential, to arboviruses evolving convergently from many taxa and lineages of ISVs. Furthermore, the accelerating rate of ISV discovery suggests that we are only seeing the tip of the iceberg with respect to these members of historic arbovirus groups.

As ISVs are nonpathogenic in vertebrates, despite their sometimes close evolutionary relationships to highly pathogenic arboviruses, there has been a growing interest in their use as tools for biological control of arbovirus transmission (Bolling *et al.*, 2015; Nasar *et al.*, 2015a). We sought to determine whether ISVs could be utilized in antigen development for vaccines and diagnostics by generating recombinant viruses with the host-restricted properties and resultant safety of ISVs, but with the antigenic properties of closely related, pathogenic arboviruses. In traditional antigen development, the pathogen of interest is manipulated in biocontainment to either attenuate it for increased safety or to completely inactivate it to abolish infectivity (Hierholzer

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et al., 1996). This results in a trade-off between reactogenicity and immunogenicity with the long-standing dogma that replicating viruses are required for rapid, robust, long-lived immunogenicity and inactivated or more contemporary subunit vaccines are safer at the expense of short-lived immunity, requiring multiple doses.

Detection and Characterization of Eilat Virus

Recently, our group characterized the first ISV within the *Alphavirus* genus of the family *Togaviridae.* Named for the city of Eilat within the Negev Desert of Israel, where mosquitoes were collected for arbovirus detection between 1982 and 1984, Eilat virus (EILV) was isolated in mosquito cells from a pool of *Anopheles coustani* by Joseph Peleg (Hebrew University, Jerusalem) and sent to Robert Tesh (University of Texas Medical Branch) for further study (Samina *et al.*, 1986). On infection of mammalian cell lines or infant mice, no evidence of pathologic changes was detected. However, infection of a mosquito cell line resulted in cytopathic effects (CPE) (Nasar *et al.*, 2012). Deep sequencing revealed the presence of EILV and a second virus, Negev virus (Vasilakis *et al.*, 2013), which was later determined to be responsible for the observed CPE in insect cells; due to its rapid replication compared to EILV, Negev virus complicated the isolation of EILV. On determining the genomic sequence of EILV, a full-length infectious clone was constructed and utilized to rescue recombinant EILV for further study (Nasar *et al.*, 2012).

Phylogenetically, EILV falls within the mosquito-borne clade of alphaviruses as a sister to the western equine encephalitis complex. EILV replicates very efficiently, producing minimal CPE, in a variety of insect cells, reaching peak titers of 5×10^8 plaque forming units (PFU)/mL within 48 h in *Aedes albopictus* mosquito cells. However, infection of a variety of vertebrate cells is blocked at both the attachment/entry and genome replication steps of replication (Nasar *et al.*, 2015b). Despite a broad insect cell host range, EILV has a very narrow *in vivo* mosquito host range (Nasar *et al.*, 2014). Perhaps the absence of a vertebrate host in the maintenance cycle of EILV facilitated its specialization on a single (mosquito) host.

Characterization of EILV Chimeras

Given the above findings, we hypothesized that a recombinant virus containing the replication machinery of EILV and the structural proteins of a pathogenic alphavirus would retain the host-restricted properties of the former and the antigenic characteristics of the latter. For our initial studies, we constructed chimeras with structural protein genes from eastern equine encephalitis virus (EEEV) and Venezuelan equine encephalitis virus (VEEV) (Erasmus *et al.*, in press), as well as chikungunya virus (CHIKV), which had recently re-emerged in the Americas to cause over 2.4 million cases of disabling arthritic disease, and rescued these viruses in mosquito cells. *In vitro*, EILV/EEEV replicates poorly compared to EILV, whereas EILV/VEEV and EILV/CHIKV replicate similarly to and better than EILV in mosquito cells, with peak titers of $\sim 10^9$ and $\sim 10^{10}$ PFU/mL, respectively. Furthermore, CPE is enhanced by the EILV/VEEV and EILV/CHIKV chimeras. This apparent gain-of-function, mediated by the structural proteins, is under further investigation. Importantly, replication in a variety of vertebrate cells could not be detected (Erasmus *et al.*, 2017).

To confirm that EILV chimeras are unable to cause disease in vertebrates, we utilized the highly susceptible newborn A129 mouse model that lacks IFN α/β receptor signaling. Intracranial infection with EILV/CHIKV at a dose of 10^9 PFU results in no significant weight loss and 100% survival (Erasmus *et al.*, 2017). Furthermore, we performed serial brain passages of EILV/CHIKV in this model and utilized plaque assays and quantitative reverse transcription PCR to quantify viral loads after each passage. After the second passage, we could no longer detect EILV/CHIKV and 100% of mice survived infection with passage 5 material, indicating that EILV/CHIKV is unable to readily adapt to a vertebrate replicative phenotype. In contrast, intracranial infection of these mice with a 10,000-fold lower dose of a live attenuated CHIK vaccine resulted in 100% mortality within 2 days, underscoring the sensitivity of this model.

We next performed experiments to determine which step in the virus replication cycle was dysfunctional in the vertebrate host, accounting for this level of safety. To evaluate attachment, we fluorescently labeled EILV/CHIKV or live attenuated CHIKV and observed no differences in the amount of virus-bound vertebrate cells by flow cytometry (Erasmus *et al.*, 2017). In contrast, formalin inactivation of EILV/CHIKV significantly reduced binding. Next, using thin-section electron microscopy, we confirmed that EILV/CHIKV enters vertebrate cells via the same receptor-mediated endocytic mechanism as pathogenic alphaviruses (Fig. 1A–C). To evaluate downstream replication events, we generated a series of reporter viruses encoding green fluorescent protein (GFP) in either the first or second open reading frames (ORFs). During alphavirus replication, the first ORF, encoding the nonstructural proteins, is translated on genome delivery, while the second ORF, encoding the structural proteins, is only translated following transcription of (1) minus-sense genomic RNA and (2) positive-sense subgenomic RNA. We observed GFP in vertebrate cells infected with EILV/CHIKV encoding GFP in the first ORF, but no GFP could be detected when encoded in the second ORF (Fig. 1D). However, both constructs expressed GFP in mosquito cells (Fig. 1E). These results suggested that, following entry, genomic RNA was delivered and translated in vertebrate cells, However, the EILV-derived nonstructural proteins were nonfunctional and unable to mediate transcription in vertebrate cells, a process that is necessary for mutation and adaptive evolution. These findings also supported our conclusion from the mouse brain passages that EILV chimeras are stably attenuated and unable to readily adapt to vertebrate hosts.

Antigenicity and Application in Diagnostic Development

To characterize the structural and antigenic properties of EILV chimeras, we performed cryoelectron microscopy, resulting in 11, 8, and 10 Å reconstructions for EILV/EEEV, EILV/VEEV, and EILV/CHIKV, respectively (Fig. 1F, G). By comparing the chimeras to their pathogenic counterparts, we concluded that EILV/VEEV and EILV/CHIKV were structurally identical to VEEV and CHIKV, respectively. As there is currently no published structure of wt EEEV, this

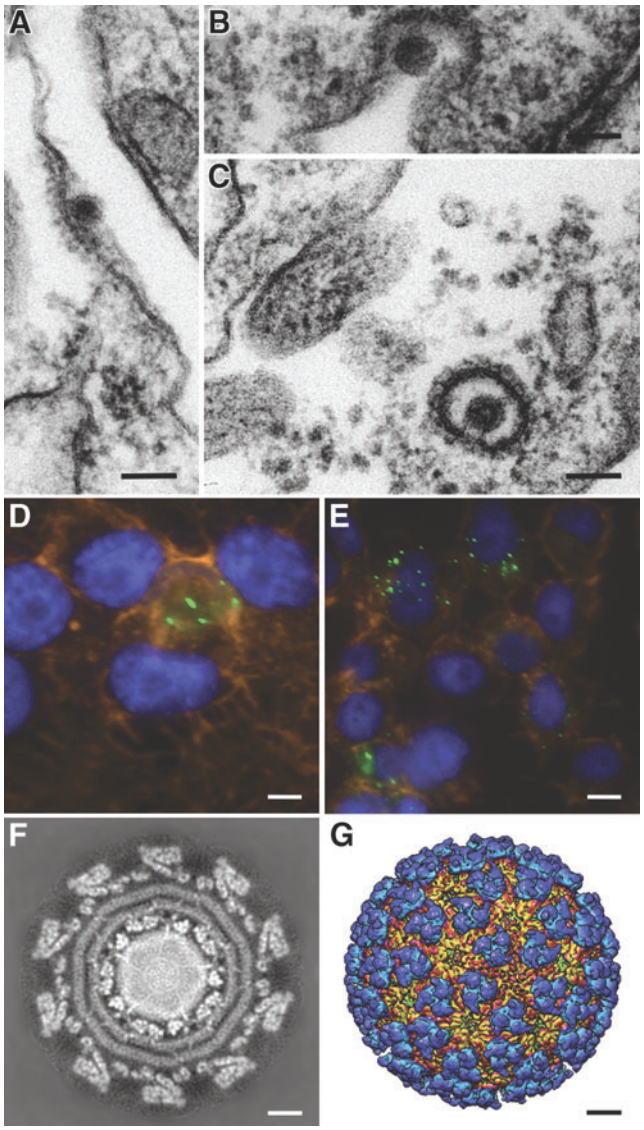

FIG. 1. Characterization of Eilat virus chimeras. Thin-section electron microscopy of Vero cells at 0 **(A)**, 15 s **(B)**, and 5 min **(C)** after infection with Eilat/chikungunya virus chimera. Confocal microscopy of Vero **(D)** or C7/10 mosquito cells **(E)** 8 h after infection with an Eilat/chikungunya chimera expressing GFP in the 5′ open reading frame. Cryoelectron microscopy reconstruction of Eilat/chikungunya virus chimera **(F, G)**. Chikungunya glycoproteins can be seen embedded in a mosquito cell-derived lipid bilayer and interacting with capsid proteins that surround a density-rich nucleic acid core **(F)**. Adapted from Erasmus *et al.* (2017) and EMDB-3406. Scale bars represent 100 nm **(A–C)**, 5 μm **(D, E)**, and 80 Å **(F, G)**. GFP, green fluorescent protein.

analysis could not be performed for EILV/EEEV. To corroborate these findings with an immunologically relevant measure, we tested the ability of EILV chimeras to bind antibodies from mice experimentally infected with EEEV, VEEV, or CHIKV. We demonstrated that EILV chimeras could indeed bind antibodies elicited by infections with their pathogenic counterparts, and the sensitivity of detection was significantly enhanced compared to formalin-inactivated antigens (Erasmus *et al.*, 2015).

In light of the ongoing CHIK outbreak in the Americas, we exploited the EILV/CHIKV ability to sensitively detect antibodies and developed ELISA-based diagnostic kits. We collected sera from CHIKV-infected patients in Chiapas, Mexico, in 2014 (Kautz *et al.*, 2015) and performed IgM ELISAs using either EILV/CHIKV as antigen or a commercially available assay that was recommended by the Centers for Disease Control and Prevention (CDC). We and others established that our assay outperformed commercial kits and resulted in 100% sensitivity and specificity (Erasmus *et al.*, 2015; Johnson *et al.*, 2016). In terms of antigen production, high-quality antigens produced by the CDC that demonstrate equivalent sensitivity and specificity require biocontainment to culture, concentrate, purify, and inactivate, which is extremely costly and time-consuming (Goodman *et al.*, 2014). Due to the safety of working with EILV/CHIKV, and the resulting low cost of production, which does not require concentration, purification, or inactivation, we rapidly translated the EILV-based chimeras from concept to a commercial product within 6 months, enabling immediate utilization by multiple public health laboratories during the CHIK epidemic.

Immunogenicity and Application in Vaccine Development

Given their antigenic properties coupled with a unique ability to attach, enter, and deliver a genome that is translated but otherwise nonfunctional in vertebrate cells, we were eager to assess the immunogenicity and efficacy of EILV chimeras as vaccines. For EILV/EEEV, /VEEV, and /CHIKV chimeras, a minimum dose of 10^8 PFU was needed to achieve single-dose efficacy in 100% of vaccines. With the exception of EILV/EEEV, this dose induced rapid neutralizing antibody induction with 100% protective efficacy in mice following lethal challenge just 4 days after immunization. With EILV/CHIKV, the most extensively evaluated, we demonstrated durable murine immunogenicity and efficacy, with high-titer neutralizing antibodies detected at least 10 months after a single dose and 100% protection against death, weight loss, viremia, and footpad swelling following CHIKV challenge (Erasmus *et al.*, 2017). When this same dose was administered in nonhuman primates, we observed similar neutralizing antibody kinetics with 100% seroconversion by day 4, and complete protection against viremia and telemetrically monitored fever after CHIKV challenge. Furthermore, all three chimeras, administered as a blended vaccine, protected murine models against EEEV and VEEV. However, immune interference was observed for the EEEV component, as neutralizing antibody titers against EEEV were significantly reduced in mice vaccinated with the blended compared to a monovalent vaccine, while anti-VEEV and anti-CHIKV neutralizing antibodies were not significantly affected by the trimeric formulation (Erasmus *et al.*, in press). The reduced immunogenicity of EILV/EEEV, and replication kinetics in mosquito cells compared to EILV/VEEV and EILV/CHIKV, deserves further study to elucidate the mechanisms of immunogenicity induced by the EILV platform.

Discussion

Some initial conclusions surrounding mechanisms of immunogenicity can be drawn from our EILV/CHIKV experiments. In the murine immunogenicity and efficacy studies, we

included as controls a dose-matched, formalin-inactivated EILV/CHIKV preparation as well as a typical dose ($\sim 10^5$ PFU) of a live attenuated CHIKV vaccine. The effects of formalin inactivation included a reduction in levels and durability of neutralizing antibodies as well as in activated $CD4^+$ and $CD8^+$ T cells. In the lethal murine challenge model of CHIKV, formalin inactivation also affected the ability to protect against weight loss and footpad swelling, despite protection from viremia and mortality (Erasmus *et al.*, 2017). Our *in vitro* characterization of formalin-inactivated EILV/CHIKV demonstrated reduced cell binding and immunodetection with polyclonal mouse anti-CHIKV sera (Erasmus *et al.*, 2015, 2017), suggesting that normal receptor-mediated endocytic uptake for effective B cell receptor engagement of antigen plays an important role as suggested by others (Cardoso *et al.*, 1995; Engering *et al.*, 1997; Arnold-Schild *et al.*, 1999; Klimstra *et al.*, 2003; Burgdorf *et al.*, 2006; Rae *et al.*, 2008; Delrue *et al.*, 2009; Kane *et al.*, 2011; Wilton *et al.*, 2014; Fan *et al.*, 2015).

In one interpretation, the differential immune response to the live attenuated CHIKV strain in the immune-competent and immunocompromised murine models may point to the role of antigen dose in the enhanced immunogenicity of EILV chimeras. While a 10^5 PFU dose of the live attenuated CHIKV strain in wild-type mice induced only partial seroconversion with a mean neutralizing antibody titer of $\sim$1:80, the same dose in IFN α/β receptor knockout mice resulted in >1:1280 titers. No differences in neutralizing antibody titers between mouse models were observed for EILV/CHIKV and its formalin-inactivated counterpart (Erasmus *et al.*, 2017). Perhaps the antiviral responses induced by replication-competent vaccines delivered at moderate doses limit replication, resulting in a suboptimal net antigen load insufficient for robust immune responses. While it is safe to administer higher doses of replication-defective viruses, the same cannot necessarily be said for replication-competent viruses (recall our comparison of EILV/CHIKV to live attenuated CHIKV in newborn mice).

In summary, while live attenuated vaccines are highly immunogenic, the replicating nature of these vaccines may not be the key to their efficacy. The minimal requirements for robust immunogenicity may be simpler: a large, but safe, antigen dose and intact early stages of the virus replication cycle, including attachment and entry. Apart from this parsimonious explanation for mechanism of immunogenicity of EILV chimeras, other more complicated explanations warrant further investigation. Perhaps the ability of EILV chimeras to deliver a genome that is subsequently translated activates pattern recognition receptors in an adjuvant-like manner. Regardless, could the ability to deliver genetic material be exploited to further enhance or to change the quality of immune responses? More work is needed to answer these questions and to test the applicability of ISVs from other families of viruses.

Disclosure Statement

J.H.E. and S.C.W. have patents related to this work.

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