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The structural proteins of epidemic and historical strains of Zika virus differ in their ability to initiate viral infection in human host cells

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ABSTRACT

Mosquito-borne Zika virus (ZIKV) recently emerged in South Pacific islands and Americas where large epidemics were documented. In the present study, we investigated the contribution of the structural proteins C, prM and E in the permissiveness of human host cells to epidemic strains of ZIKV. To this end, we evaluated the capacity of the epidemic strain BeH819015 to infect epithelial A549 and neuronal SH-SY5Y cells in comparison to the African historical MR766 strain. For that purpose, we generated a molecular clone of BeH819015 and a chimeric clone of MR766 which contains the BeH819015 structural protein region. We showed that ZIKV containing BeH819015 structural proteins was much less efficient in cell-attachment leading to a reduced susceptibility of A549 and SH-SY5Y cells to viral infection. Our data illustrate a previously underrated role for C, prM, and E in ZIKV epidemic strain ability to initiate viral infection in human host cells.

1. Introduction

Zika virus (ZIKV) is an emerging mosquito-borne *flavivirus* (*Flaviviridae* family) that became a major medical concern worldwide. In 2007, the first ZIKV epidemic occurred in the Yap Island affecting more than 70% of the inhabitants (Duffy et al., 2009). Subsequently, ZIKV continued to spread in the South Pacific islands and widely emerged in the Americas from 2015 (Gatherer and Kohl, 2016). Phylogenetic analysis of viral sequences identified the African and Asian genotypes of ZIKV (Haddow et al., 2012), the Asiatic strains being the leading cause of the major current epidemics with millions of infection cases (Giovanetti et al., 2016). While a large majority of infections caused by ZIKV are asymptomatic, or result in dengue-like symptoms, epidemiological studies have pointed out that ZIKV infection can also cause severe diseases in humans including Guillain-Barre syndrome and congenital neurological defects (microcephaly) (Cao-Lormeau et al., 2016; Cugola et al., 2016; Merfeld et al., 2017; Parra et al., 2016). Unlike most other flaviviruses, a component of the spread of ZIKV may reflect its potential for human-to-human transmission. ZIKV is believed to disseminate using both sexual and non-sexual routes including body fluids such as tears, saliva or blood (D'Ortenzio et al., 2016; Motta et al., 2016; Musso et al., 2016). The risk of human infection by solid organ transplantation has also been suggested (Alcendor, 2017).

ZIKV contains a single-strand positive sense genomic RNA, which is translated into a large and unique polyprotein. The polyprotein is then processed by host and viral proteases into three structural proteins (C, prM/M and E), and seven nonstructural (NS) proteins (NS1 to NS5). Recently, we reported the generation of an infectious cDNA clone of MR766 (hereafter MR766^{MC}) based on the GenBank access number LC002520 (MR766-NIID) after electroporation of four overlapping synthetic fragments that cover the genomic RNA sequence using the ISA method (Atieh et al., 2016; Gadea et al., 2016). The African historical ZIKV strain MR766 was initially collected from a rhesus monkey in Uganda in 1947 and serially propagated in new-born mouse brains (Dick et al., 1952). It is not known whether the mouse neuroadaptation of MR766 resulted in accumulation of mutations in viral sequence.

Epidemic strains of ZIKV have the capacity to replicate in diverse cell types of human origin including epithelial and neuronal cells (Hamel et al., 2017; Pagani et al., 2017; Sheridan et al., 2017; Simonin et al., 2016; Tripathi et al., 2017). We reported that infection of human lung epithelial A549 cells with ZIKV strain PF-25013-18 of Asian lineage isolated during the epidemic in French Polynesia in 2013 (Cao-Lormeau et al., 2014; Frumence et al., 2016) resulted in the activation of Interferon Stimulated Genes (ISGs), the production of Type-I

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interferon (IFN) associated to expression of pro-inflammatory cytokines, and induction of apoptosis. Several reports highlighted the preponderant role of the NS proteins in the responsiveness of human host cells to ZIKV infection. Here, we studied the role of the structural proteins in the permissiveness of human neuroblastoma SH-SY5Y and A549 cells to infection with epidemic strains of ZIKV. Using the ISA method, we generated a molecular clone of ZIKV strain BeH819015 isolated from a human serum specimen in Brazil in 2015 and a chimeric clone of MR766 in which the structural protein region was replaced with the one of BeH819015. Comparative analysis between the three viral clones revealed that ZIKV containing BeH819015 structural proteins are less efficient in the initiation of viral infection in human cells when compared to $MR766^{MC}$.

2. Methods

2.1. Cells and reagents

Vero cells (ATCC, CCL-81) were cultured at 37 °C under a 5% CO2 atmosphere in MEM medium, supplemented with 5% heat-inactivated foetal bovine serum (FBS), A549-Dual™ cells (InvivoGen, a549d-nfis) designated hereafter as $A549^{DUAL}$ cells and SH-SY5Y cells (ATCC, CRL2266) in MEM medium, supplemented with 10% heat-inactivated FBS and non-essential amino acids. A549-Dual™ ($A549^{Dual}$) cells were maintained in growth medium supplemented with 10 μg mL^{-1} blasticidin and 100 mg mL^{-1} zeocin (InvivoGen). The mouse anti-pan flavivirus envelope E protein mAb 4G2 was produced by RD Biotech. The rabbit anti-BAX and anti-caspase 3 antibodies were purchased from Cell Signalling Technology, the anti-cleaved PARP antibody from Promega, donkey anti-mouse Alexa Fluor 488 and anti-rabbit Alexa Fluor 594 IgG antibodies from Invitrogen. Horseradish peroxidase-conjugated anti-rabbit and anti-mouse antibodies were purchased from Vector Labs.

2.2. Design of ZIKV molecular clones

The molecular clone design and production strategies for ZIKV were previously described by Gadea et al. (2016). The viral clone $BR15^{MC}$ was produced as described for $MR766^{MC}$ (Gadea et al., 2016). $BR15^{MC}$ was based on the sequence of epidemic ZIKV strain BeH819015 (GenBank access KU365778) isolated in Brazil in 2015. MR766 was passaged more than hundred times in new-born mouse brain, and BeH819015 was isolated from blood patient and sequenced after one passage on C6/36 (Dick et al., 1952; Faria et al., 2016). Because the sequences of the untranslated regions (UTRs) deposited in GenBank were partial and also showed abnormalities, the 5'UTR of $MR766^{MC}$ and the 3'UTR of contemporary clinical isolate Paraiba of ZIKV (GenBank access KX280026) were chosen to generate $BR15^{MC}$. The 3'UTRs from $BR15^{MC}$ and $MR766^{MC}$ perfectly matched on the last 150 nucleotides. Based on our previous experience with molecular clone of ZIKV that used overlapping PCR products (Gadea et al., 2016), the design of viral genome into four viral genomic fragments $Z1^{BR15}$, $Z2^{BR15}$, $Z3^{BR15}$ and $Z4^{BR15}$ was chosen to mimic those used to construct $MR766^{MC}$. The fragment $Z1^{BR15}$ contains the CMV promoter immediately adjacent to the 5'UTR of ZIKV followed by the structural protein region of strain BeH819015. Two synonymous mutations at positions 2200 (a - > g) and 2215 (t - > c) of the structural protein region of ZIKV strain BeH819015 were introduced for a perfect matching of the fragments $Z1^{BR15}$ and $Z2^{NIID\text{-}MC}$ on 42 nucleotides. A silent mutation in the E gene that creates the unique restriction site *Pvu* I was introduced into the $Z1^{BR15}$ fragment. The fragment $Z4^{BR15}$ consists of a hepatitis delta virus ribozyme immediately following the last ZIKV nucleotide and a SV40 poly(A) signal. The synthetic genes $Z1^{BR15}$, $Z2^{BR15}$, $Z3^{BR15}$, and $Z4^{BR15}$ were synthesized and cloned into plasmid pUC57 by GeneCust (Luxembourg). The fragments $Z1^{BR15}$ to $Z4^{BR15}$ were amplified by PCR from their respective plasmids using a set of primer pairs that were designed so that $Z1^{BR15}$ and $Z2^{BR15}$ or $Z3^{BR15}$ and $Z4^{BR15}$ matched on about 30–40 nucleotides.

2.3. Recovering of molecular clone $BR15^{MC}$ and chimeric virus CHIM

The molecular clone $BR15^{MC}$ was produced as previously described for $MR766^{MC}$. Briefly, the purified PCR fragments $Z1^{BR15}$, $Z2^{BR15}$, $Z3^{BR15}$, and $Z4^{BR15}$ were electroporated into Vero cells. After 5 days, cell supernatants were recovered and used to infect fresh Vero cells in a first round of amplification (P1). Viral clone $BR15^{MC}$ was recovered 7 days later and amplified for another 2 days on Vero cells to produce a P2 for further studies. The viral clone $BR15^{MC}$ derived from ZIKV strain BeH819015 is available to BEI Resources (Manassas, VA) under the catalog number NR-51129 (www.beiresources.org). To produce the viral clone CHIM, Vero cells were electroporated with the PCR fragments $Z1^{BR15}$, $Z2^{MR766\text{-}MC}$, $Z3^{MR766\text{-}MC}$, and $Z4^{MR766\text{-}MC}$. The recovered virus CHIM consists of the viral sequence of MR766 in which the coding region for the structural proteins was replaced with the counterpart of ZIKV strain BeH819015. The viral clone CHIM contains a chimeric E protein between BeH819015 (amino acids 1–436) and MR766 (amino acids 437–504).

2.4. Plaque forming assay

Viral titers were determined by a standard plaque-forming assay as previously described with minor modifications (Frumence et al., 2016). Briefly, Vero cells grown in 48-well culture plate were infected with tenfold dilutions of virus samples for 2 h at 37 °C and then incubated with 0.8% carboxymethylcellulose (CMC) for 4 days. The cells were fixed by 3.7% FA in PBS and stained with 0.5% crystal violet in 20% ethanol. Viral titers were expressed as plaque-forming units per mL (PFU.mL^{-1}).

2.5. Quantification of viral stocks

Zika virus samples were analyzed by titration on Vero cells while genomic viral RNA was quantified by RT-qPCR. For viral genome quantification, viral RNA was extracted from virus particles using QIAmp kit (QIAGEN). The PCR standard curve used for the quantification of ZIKV copy numbers was obtained with a pUC57/ZIKV-E amplicon plasmid containing a synthetic cDNA encompassing nucleotides 961–1301 of genomic RNA (GenBank accession number LC002520). The couple of ZIKV E primers was used to equally amplify pUC57/ZIKV-E amplicon and the cDNA encompassing nucleotides 1046–1213 from genomic RNA of ZIKV molecular clones used in this study.

2.6. Immunoblot assay

Cell lysates were performed in RIPA lysis buffer. All subsequent steps of immunoblotting was performed as described (Nativel et al., 2013; Viranaicken et al., 2011). Primary antibodies were used at 1:500 dilutions. Anti-rabbit immunoglobulin-horseradish peroxidase and anti-mouse immunoglobulin-horseradish peroxidase conjugates were used as secondary antibodies (dilution 1:2000). Blots were revealed with ECL detection reagents. Bands were quantified by densitometry using ImageJ software.

2.7. Immunofluorescence assay

$A549^{Dual}$ cells grown on glass coverslips were fixed with 3.7% formaldehyde at room temperature for 10 min. Fixed were permeabilized with 0.1% Triton X-100 in PBS for 4 min. Cells were stained using the mouse anti-pan flavivirus envelope E protein mAb 4G2 (1:1000 dilution), rabbit anti-Caspase 3 mAb (1:1000 dilution) and rabbit anti-BAX mAb (1:1000 dilution). Antigen staining was visualized with Alexa Fluor-conjugated class specific secondary antibodies (1:1000, Invitrogen). Nucleus morphology was revealed by DAPI staining. The