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Research Paper

Zika Virus Strains Potentially Display Different Infectious Profiles in Human Neural Cells

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ABSTRACT

The recent Zika virus (ZIKV) epidemic has highlighted the poor knowledge on its physiopathology. Recent studies showed that ZIKV of the Asian lineage, responsible for this international outbreak, causes neuropathology *in vitro* and *in vivo*. However, two African lineages exist and the virus is currently found circulating in Africa. The original African strain was also suggested to be neurovirulent but its laboratory usage has been criticized due to its multiple passages. In this study, we compared the French Polynesian (Asian) ZIKV strain to an African strain isolated in Central African Republic and show a difference in infectivity and cellular response between both strains in human neural stem cells and astrocytes. Consistently, this African strain led to a higher infection rate and viral production, as well as stronger cell death and anti-viral response. Our results highlight the need to better characterize the physiopathology and predict neurological impairment associated with African ZIKV.

1. Introduction

The current (2015–2016) Zika virus (ZIKV) epidemic in South America and in the Caribbean islands revealed the lack of knowledge on the pathophysiological mechanisms of a virus discovered almost 70 years ago in Uganda (Dick et al., 1952). This arbovirus is transmitted by vectors from the *Aedes* family, in particular *Aedes aegypti* and *Aedes albopictus*. Phylogenetic analyses revealed recently that three lineages (one Asian and two Africans) exist and explain the evolution of ZIKV over the last 70 years (Shen et al., 2016; Gong et al., 2016). The current epidemic is due to ZIKV from the Asian lineage, even though some further genetic evolution seems to have occurred (Wang et al., 2016).

While a large proportion of infected persons are asymptomatic (70–80%), others develop rather classical clinical signs of an arboviral infection namely, skin rash, headache, myalgia, joint pain, conjunctivitis, but also moderate fever. In some cases, neurological disorders have been linked to ZIKV infections, in particular Guillain-Barré syndrome (GBS), myelitis, encephalitis, neuralgia and microcephaly in newborns and infants born to women with ZIKV infection during pregnancy (Musso et al., 2016). Microcephaly is now well documented as a direct disorder triggered by ZIKV infection, as the virus can cross the placenta (Miner et al., 2016), is found in the amniotic fluid (Calvet et al., 2016) and can be detected in post-mortem newborn brains (Brasil Martines et al., 2016). In particular, infection during the first trimester of pregnancy has been proposed to lead to microcephaly in 1% of cases (Cauchemez et al., 2016). Studies on animal models (rodent) show that ZIKV can cause a similar pathology as in humans, such as trans-placental fetal transmission and neurological impairment in fetal and adult brain (Rossi et al., 2016; Dowall et al., 2016; Aliota et al., 2016; Cugola et al., 2016; Lazear et al., 2016; Miner et al., 2016). However, most of these models are based on an immunodeficient background. Recent reports clearly demonstrate that ZIKV can infect human and murine neural precursors. *Ex vivo* works using induced pluripotent cells (IPSc)-derived neural precursors cells (NPCs) and neural stem cells (NSCs) show that ZIKV has a preferential tropism for cells of the neuronal lineage during development (Tang et al., 2016; Qian et al., 2016; Garcez et al., 2016). Notably, some reports suggest that impairment of

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proliferation and cell death is associated with ZIKV infection (Tang et al., 2016; Li et al., 2016), whereas another report suggests that ZIKV poorly stimulates immune response and cytopathic effect in human neuroprecursors obtained from fetal tissue (Hanners et al., 2016). This lack of strong immune response would allow ZIKV to persist during development and is consistent with the replication seen over few weeks in this tissue. These observations are strengthening the hypothesis that infection early during brain development can have drastic effects.

While most of the focus has been directed to Asian ZIKV strains or to African MR-766, much less effort has been undertaken to monitor potential circulating ZIKV of the African lineage (Grard et al., 2014; Baraka and Kweka, 2016; Meda et al., 2016). In this context, there is an urgent need to have clear understanding of the pathophysiological mechanisms involved in infection by African ZIKV, in particular in terms of neurovirulence. In other words, to know whether the neurological effects observed with the Asian lineage are specifically associated with the Asian strain, in terms of severity and specificity, or if we can expect African strains to lead to similar disorders. So far, most of the studies that investigated African ZIKV strain used the original strain of ZIKV (MR-766, isolated in 1947). Lately, criticisms emerged concerning the pertinence of this strain, isolated from primates and extensively amplified in suckling mouse brains and *ex vivo* on cells (Haddow et al., 2012; Musso et al., 2016).

Here, we used IPSc-derived human NSCs to better compare the neural infectivity of an Asian strain (ZIKV AS) and an African strain (ZIKV AF) that underwent low passages. We demonstrate that this ZIKV AF strain is more infectious than the French Polynesian ZIKV AS PF-13 strain (H/PF/2013): indeed, this strain showed a higher rate of infection, viral production and cellular response (cell death and anti-viral response) than ZIKV AS. Finally, we show that ZIKV AF and AS strains also display difference in infection of human astrocytes.

2. Material and Methods

2.1. Material

Antibodies used in this study are: anti-pan-flavivirus (MAB10216, clone D1-4G2) and anti-nestin, (Millipore), anti-GFAP (Abcam), anti-PDI and anti-activated caspase 3 (Cell Signalling Technology), anti-TRA1-60 (Becton Dickinson) and anti-PAX6 (BioLegend). Carboxyfluorescein succinimidyl ester (CFSE) dye was purchased from Thermoscientific.

2.2. ZIKV Strains, Production and Cellular Infection

H/PF/2013 ZIKV of Asian lineage (French Polynesia, 2013) and ArB41644 ZIKV of African lineage (Bangui, Central African Republic, 1989, isolated from mosquitoes by Pasteur Institute of Dakkar) were produced and provided by the National Reference Center for arboviruses (NRC) and have both no >5 passages on Vero cells. Viral stocks were prepared by infecting sub confluent Vero cells at the multiplicity of infection (MOI) of 0.01 in D-MEM medium (Thermoscientific) supplemented by 2% heat-inactivated fetal bovine serum (Sigma). Cell supernatant was collected 6 days later and viral stock harvested after centrifugation at 300*g* to remove cellular debris. Viral titers were determined by the 50% tissue culture infective dose (TCID50), which was calculated using the Spearman-Kärber method (Kärber, 1931) and were expressed as TCID50 per mL. Titers were calculated twice, once at the NRC and once in our laboratory. Another stock from each ZIKV strain was also produced in C6/36 cells and had similar results (data not shown).

IPSc-derived NSCs and human astrocytes (see below) at 60–70% confluence were rinsed once with phosphate-buffered saline (PBS), and ZIKV diluted to the required MOI (0.01, 0.1 or 1) was added to the cells in a low medium volume. Cells were incubated for 2 h at 37 °C with permanent gentle agitation and then the inoculum was removed and cells washed with PBS. Culture medium was added to each well, and cells were incubated at 37 °C and 5% CO_2. As control, cells were incubated with the culture supernatant from Vero cells (mock condition).

2.3. NSC Generation and Maintenance

NSCs were obtained from the SAFE-IPSc platform at IRMB. Briefly, iPSCs generated from healthy patient using Lentivirus-derived vectors were individualized with Gentle Cell Dissociation Reagent (Stemcell, 07174). They were rinsed out with Dulbecco's modified Eagle's medium/Ham's F12 (DMEM/F-12, Gibco, 31330038) and centrifuged at 300*g* for 5 min. Dissociated cells were plated on matrigel at a density of 20,000–40,000 cells/cm^2 and cultured in neural induction medium (Stemcell, 05835) supplemented with 10 μM ROCK-inhibitor (Y-27632). Cells were allowed to reach 80–90% confluence over 6 days. Medium was changed daily with neural induction medium without Y-27632. iPSC-derived NSCs were passaged by incubation with Trypsin at 0.005% to allow dissociation, and then seeded on poly-D-ornithine/laminin coated plates at 20,000 cells/cm2 in 50% DMEM/F-12 and 50% Neurobasal medium (Thermoscientific) supplemented with 1× N2 (Thermoscientific), 1× B27 (Thermoscientific), Glutamax (Thermoscientific) and β-FGF plus EGF (Peprotech, 20 ng/mL each). The medium was changed every two days. Cells were used between passages 5 and 8.

2.4. Astrocyte Cultures

Astrocytes were purchased from ScienCell™ and cultured according to the manufacturer's instruction. Cells were cultured on Poly-lysine coated plates and were used between passages 3 and 7.

2.5. Immunofluorescence Assays

NSCs plated on poly-D-ornithine/laminin coverslips and astrocytes plated on poly-D-lysin coverslips were imaged using Zeiss SP8 confocal microscope, with 40× or 63× 1.4 NA Plan Apochromat oil-immersion objectives. For indirect immunofluorescence, cells were fixed with 4% PFA and permeabilized with 0.1% Triton X-100/PBS for 5 min at room temperature (RT), followed by a blocking step with 2% bovine serum albumin (BSA) and 10% horse serum for 30 min to 1 h at RT. Primary and secondary antibodies were diluted in blocking solution and incubated sequentially for 1 h at RT. Samples were then mounted with fluorescent mounting medium (Prolongold, Thermofischer) with DAPI (Sigma) and imaged by confocal microscopy using the Zeiss SP8 confocal microscope, with 40× or 63× 1.4 NA Plan Apochromat oil-immersion objectives. Nuclei and ZIKV infected cells were counted using a plugin of the Image J software.

2.6. Flow Cytometry and CFSE Assays

NSCs were incubated with CFSE according to the manufacturer (Thermoscientific) for 3 min at RT after enzymatic dissociation and replated after the dye was quenched and cells were washed with PBS. NSCs infected with ZIKV strains were collected in PBS at various days, washed with PBS, fixed with 2% PFA and analyzed with FACSCalibur (BD Biosciences). Data analysis was performed using the FlowJo software. Decrease in fluorescence intensity is consistent with cell division.

2.7. RT-qPCR

RT-PCR on ZIKV-infected NSC supernatants was performed after mRNA extraction with the Altona Diagnostics kit RealStar® Zika Virus RT-PCR Kit 1.0, according to the manufacturer's instructions. Experiments done with similar MOI for ZIKV AF and AS showed equal amplification for both strains (data not shown). For PCR arrays, NSCs infected with ZIKV AF or AS or mock-treated cells were harvested in RLT buffer

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