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Research Paper

American Strain of Zika Virus Causes More Severe Microcephaly Than an Old Asian Strain in Neonatal Mice

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ABSTRACT

Zika virus (ZIKV) has evolved from an overlooked mosquito-borne flavivirus into a global health threat due to its astonishing causal link to microcephaly and other disorders. ZIKV has been shown to infect neuronal progenitor cells of the fetal mouse brain, which is comparable to the first-trimester human fetal brain, and result in microcephaly. However, whether there are different effects between the contemporary ZIKV strain and its ancestral strain in the neonatal mouse brain, which is comparable with the second-trimester human fetal brain, is unclear. Here we adopted a mouse model which enables us to study the postnatal effect of ZIKV infection. We show that even 100 pfu of ZIKV can replicate and infect neurons and oligodendrocytes in most parts of the brain. Compared with the ancestral strain from Cambodia (CAM/2010), infection of the ZIKV strain from Venezuela (VEN/2016) leads to much more severe microcephaly, accompanied by more neuronal cell death, abolishment of oligodendrocyte development, and a more dramatic immune response. The serious brain damage caused by VEN/2016 infection would be helpful to elucidate why the American strain resulted in severe neurovirulence in infants and will provide clinical guidance for the diagnosis and treatment of infection by different ZIKV strains.

1. Introduction

The World Health Organization has declared the current ZIKV epidemic a Public Health Emergency of International Concern due to the causal link between the current Zika strain and congenital brain abnormalities, especially microcephaly in the fetuses and offspring of pregnant women (Rubin et al., 2016; Parra et al., 2016). Clinical investigations and animal models have provided substantial evidence that ZIKV can infect cultured neuronal progenitor cells (NPCs) and organoids from human iPSCs, or directly target mouse, pigtail macaque or human fetal brains, cause neural cell death, and lead to fetal brain lesions and microcephaly (Li et al., 2016a; Tang et al., 2016; Wu et al., 2016; Miner et al., 2016; Garcez et al., 2016; Rasmussen et al., 2016; Adams Waldorf et al., 2016).

Several recent studies have confirmed the causal link between ZIKV infection and microcephaly in embryonic mouse models infected with ZIKV (Li et al., 2016b; Wu et al., 2016; Shao et al., 2016). Contemporary ZIKV strains are able to infect the neuronal progenitor cells of fetal brains and disrupt their development including proliferation and differentiation, trigger a strong immune response, cause neuronal cell death and microcephaly in the embryonic mouse brain (Tang et al., 2016; Li et al., 2016b; Li et al., 2016c; Wu et al., 2016; Rasmussen et al., 2016). However, ZIKV was an obscure mosquito-borne flavivirus which circulated for decades in Sub-Saharan Africa and Asia since its first isolation 60 years ago (Mlakar et al., 2016; Hayes, 2009). Retrospective evidence of microcephaly in newborn patients was retrieved only after 2013–14 in French Polynesia and later in the Americas (Duffy et al., 2009; Cao-Lormeau et al., 2014). Therefore, several critical questions remain open regarding whether different ZIKV strains have different detrimental effects on brain development, and whether the contemporary ZIKV

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strains have evolved to become more neurotropic with increased replicative capacity which then plays a role in the development of microcephaly.

The CAM/2010 strain is an old Asian strain of ZIKV isolated from a Cambodia patient in 2010 (Shan et al., 2016; Haddow et al., 2012) and is not associated with human severe neuronal disorder or microcephaly in infants in the past few years (Heang et al., 2012). The VEN/2016 strain, meanwhile, is a contemporary American strain isolated from a patient returning from Venezuela in 2016 (Zhang et al., 2016b; Li et al., 2016d). There are some amino acid variations between the CAM/2010 and VEN/2016 strains (Table S1). Since the neonatal mouse brain is relatively immature, similar to that of a second-trimester human fetus (Ornaghi et al., 2017), and ZIKV infection at this stage of pregnancy has been reported to cause microcephaly (Brasil et al., 2016; Franca et al., 2016), we adopted a neonatal mouse brain infection model to compare the effects of ZIKV CAM/2010 and VEN/2016 strains in the neonatal mouse brain (Huang et al., 2016). In this study, we found that VEN/2016 infection led to much more severe microcephaly than CAM/2010, accompanied by loss of more neurons and oligodendrocytes, and a much stronger immune response.

2. Methods and Materials

2.1. Ethics Statement

All animal work was conducted strictly according to the guidelines of the Chinese Regulations of Laboratory Animals (Ministry of Science and Technology of People's Republic of China) and Laboratory Animal-Requirements of Environment and Housing Facilities (GB 14925-2010, National Laboratory Animal Standardization Technical Committee). All of the procedures were approved by the Animal Experiment Committee of Laboratory Animal Center, Academy of Military Medical Sciences (AMMS), China (IACUC-13-2016-001).

2.2. Animals and ZIKV Inoculation

100 pfu of ZIKV (CAM/2010, an old Asian strain or VEN/2016, a South America strain) or PBS buffer (20 μL) was injected into the λ point of ICR mouse brains at postnatal 0 day and the infected brains were inspected at different time points as indicated. The mice were provided by the Laboratory Animal Center, AMMS, China. The experimental procedures on mice were performed under biosafety level 2 (BSL-2) at Beijing Institute of Microbiology and Epidemiology with Institutional Biosafety Committee approval (Li et al., 2016b).

2.3. Virus Preparation

The ZIKV stocks (both VEN/2016 and CAM/2010 strains) were from the Virus Bank of Beijing Institute of Microbiology and Epidemiology, and the isolation and characterization were performed in Cheng-Feng Qin's laboratory as described previously (Deng et al., 2016; Li et al., 2016d). The VEN/2016 of ZIKV (GenBank accession NO. KU820898) was isolated from the acute-phase serum of a patient infected with ZIKV who returned from Venezuela to China in 2016 (Li et al., 2016d). The CAM/2010 (GenBank number KU955593), the old Asian strain, was isolated from a patient in Cambodia in 2010 (Shan et al., 2016). The initial virus seeds were passaged for three rounds of serial-propagations in cultured *Aedes albopictus* C6/36 cells, purchased from the American Type Culture Collection (ATCC, NO. CRL-1660). The virus titers were measured based on the standard plaque assay on BHK-21 cells which were purchased from ATCC (NO. CCL-10), and the virus was stored at −80 °C in aliquots before used.

2.4. Immunostaining

Immunostaining was performed as described previously (Zhang et al., 2016a). Briefly, after the dissected brains were fixed in PFA (4%) and then dehydrated in sucrose (30%), these brains were frozen and sectioned into 40 μm slices using with Leica CM1950. The brain slices were first incubated in blocking buffer (10% FBS, 5% BSA, 0.3% Triton X-100, 0.01% NaN3 dissolved in PBS) at room temperature for 1 h, and then incubated in primary antibodies dissolved in blocking buffer at 4 °C for at least 8 hours, and finally incubated in fluorescence-conjugated secondary antibodies at RT for 1 h. The antibodies used for immunostaining: ZIKV anti-serum (1:1000) (Li et al., 2016a), cleaved-Caspase3 (abcam, ab2302, 1:1000), GFAP (abcam, ab7260, 1:1000), NeuN (abcam, ab104224, 1:1000), Calbindin (Millipore, ab1778, 1:200), MBP (abcam, ab7349, 1:100), CNPase (abcam, ab6319, 1:200), CD68 (abcam, ab125212, 1:500), Iba1 (abcam, ab5076, 1:500), Sox2(abcam, ab97959, 1:1000), Ki67 (abcam, ab15580, 1:1000), Olig2 (Millipore, ab9610, 1:1000).

2.5. Toluidine Blue Staining

Brain slices were stained with 0.1% toluidine blue for 30 min, dehydrated in turns by 70%, 95% and 100% ethanol (45 s, twice for each). Slices were then hyalinized by Xylene for 10 min before sealed with neutral balsam.

2.6. Alizarin Red S staining

Brain slices were incubated with 2% Alizarin Red S (pH: 4.1–4.3) at RT for 2 min, and washed in PBS for 10 min for three times.

2.7. RNA Extraction and Real-time PCR

The RNA extraction experiment was performed with PureLink® RNA Mini Kit (Thermo Fisher Scientific) according to manufactory's protocol. The Real-time PCR were performed as described previously (Schmittgen and Livak, 2008). The sequence of primers used for detecting the RNA of ZIKV was: 5′-CCGCTGCCCAACACAAG-3′ (F) and 5′-CCAC TAACGTTCTTTTGCAGACAT-3′ (R). The primer sequences used for the immune factors were shown in Table S2.

2.8. Imaging and Statistics Analysis

Confocal imaging and quantification were performed as described previously (Zhang et al., 2014). The confocal images were obtained by the Zeiss LSM700 confocal microscopy, and Leica MZ16F Stereomicroscope was used to achieve the images of Toluidine blue staining and Alizarin Red S staining. These images were analyzed with the ImageJ, Photoshop and Imaris software. For statistical analysis, *t*-test or One-Way ANOVA with Tukey's multiple comparisons test were employed to analyze the data using Prism software, and the significant difference was indicated as $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$. n, number of brains analyzed. The microcephaly is defined by smaller brain sizes, enlarged lateral ventricles, and thinner cortex than controls.

3. Results

3.1. ZIKV infection by the American strain leads to more severe microcephaly than the old Asian strain

ZIKV infection at the second trimester of pregnancy in women can cause brain abnormality in children, and the neonatal mouse brain is comparable with the brain of second-trimester human fetus (Franca et al., 2016; Brasil et al., 2016). In order to investigate the effect of ZIKV infection at this stage of brain development, we injected 100 pfu

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