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Research Paper

Infectivity of Immature Neurons to Zika Virus: A Link to Congenital Zika Syndrome

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ABSTRACT

Background: Epidemiological data strongly suggest that microcephaly cases in Brazil are associated with the ongoing epidemic of Zika virus (ZIKV). In order to further solidify the possible link, we investigated the infectivity of ZIKV using various neuroblastoma (NB) cell lines.
Methods: Six undifferentiated, two terminally differentiated and two retinoic acid (RA) –induced, partially differentiated cell lines were exposed to ZIKV strain PRVABC59, which is genetically similar to the French Polynesia strain, with 97–100% genetic homology to the current ZIKV strain found in Brazil. All infections were confirmed by real-time PCR (RT-qPCR), immunofluorescence assay (IFA) probing with anti-flavivirus E antibody, and evaluation of cytopathic effects.
Findings: ZIKV infected all six undifferentiated NB cell lines. In five out of six NB cell lines, between 90 and 70% cells were positive by IFA whereas for one cell line, CCL-127, ~80% of cells were positive for ZIKV as determined by IFA but showed persistent infection. Two differentiated cell lines, JFEN and T-268, were highly resistant to ZIKV with <1% of the cells being susceptible, as determined by IFA and confirmed by qRT-PCR. Two retinoic acid (RA)-induced NB partially differentiated cell lines showed no difference in permissiveness as compared to their undifferentiated mother cell lines.
Interpretation: These findings strengthen the reported association between high incidences of microcephaly and ZIKV infection in newborns in Brazil. Our results suggest that the undifferentiated neurons are highly permissive to ZIKV infection, as one would expect during the early stages of neurogenesis in fetal brains; whereas differentiated neurons, representative of adult brain neurons, are relatively resistant to the virus, which explains the rare occurrence of neurological complications in adults infected with ZIKV. Our studies confirm the neurotropism of the ZIKV strain closely related to the current epidemic in Latin America.

1. Introduction

Zika virus (ZIKV) is a positive-sense, single-stranded RNA member of the family *Flaviviridae* (Mlera et al., 2014). The natural transmission cycle of ZIKV predominantly involves mosquito vectors from the *Aedes* genus and monkeys (Hayes, 2009), although ZIKV antibodies have been discovered in a number of other animals (Henderson et al., 1968; Kaddumukasa et al., 2015), including rodents (Darwish et al., 1983). Humans generally act as infrequent hosts (Kaddumukasa et al., 2015), but may represent a primary amplification and reservoir species in endemic areas (Darwish et al., 1983). The first report of clinical manifestations of ZIKV was incidental to an outbreak of jaundice in Afikpo, Nigeria in 1954, which was suspected of being yellow fever (MacNamara, 1954). The virus causes an acute febrile illness, symptomatically similar to dengue, and is characterized by mild headache, fever, maculopapular rash, joint and back pain, and general malaise, sometimes accompanied by conjunctival hyperemia, anorexia, dizziness, diarrhea and constipation (Dick et al., 1952; Bearcroft, 1956; Simpson, 1964). The incubation period is between 3 and 12 days and symptoms may last for 2–7 days. Only ~20% of presenting patients infected with ZIKV exhibit symptoms and the virus has never been reported to cause hemorrhagic fever or death, often resulting in misdiagnosis as dengue (Dick et al., 1952; Bearcroft, 1956; Simpson, 1964).

In early 2015, a serious ZIKV outbreak was recorded in Camaçari, Bahia, Brazil (Plourde and Bloch, 2016; Cerbino-Neto et al., 2016) and this was later followed by similar illnesses in five neighboring states leading the Ministry of Health to issue a ZIKV alert in April (Rasmussen et al., 2016; Chan et al., 2016). By the end of the year the virus had spread through 19 states, many in the northeastern part of the country. Subsequently, the virus migrated throughout most other South and Central American countries (Chan et al., 2016) and the pandemic swept into the Caribbean with autochthonous transmission in

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at least six island states. The rapid movement of ZIKV, together with troubling reports of association between ZIKV and infants born with microcephaly, prompted the WHO to issue an "International Health Emergency" on February 1, 2016. Nevertheless, a direct relationship between ZIKV infection and increased incidence of neurologic disorders and microcephaly have emerged, and now there is irrefutable evidence to support a strong link between ZIKV infection and the observed neurologic defects in newborns (reviewed in 12–14).

Until recently, there have been few reports demonstrating a direct link between ZIKV infection and congenital Zika syndrome (CZS). Of three new studies, the recovery of a full length ZIKV genome from an infant with microcephaly, recovery of ZIKV or positive RNA from amniotic fluid of an abnormal fetus, and recovery from brain tissues of two fetuses from ZIKA infected mothers provide convincing evidence for such a link (reviewed in 12–13). Moreover, in certain instances, impacted regions of the Brazilian outbreak registered a >10-fold increase in occurrence of microcephaly; a magnitude surge that cannot be explained by random clustering. Earlier ZIKV outbreaks in French Polynesia too have been retrospectively strongly associated with increased occurrence of microcephaly (European Centre for Disease Prevention and Control (ECDC), 2015).

A major cause for concern is that ZIKV and related viruses may adapt to transmission through species of mosquito other than *Aedes aegypti*. For example, *Aedes albopictus* is well established around the Mediterranean in Europe and is present in at least 32 states in the US (Cauchemez et al., 2016). This species could provide a vector for the onward transmission of ZIKV across both continents that receive viremic returning travelers (Zammarchi et al., 2015), since there is laboratory evidence that *A. albopictus* is a competent vector for the virus (Wong et al., 2013). Moreover, changes in climate may contribute to range expansion for ZIKV and its vectors (Kraemer et al., 2015), and autochthonous cases in temperate regions may occur even in absence of a competent vector through sexual transmission (Venturi et al., 2016).

At present, no specific vaccines or treatments for ZIKV are available and, although the possibility exists that present flavivirus vaccine technologies could be adapted to ZIKV (Rochlin et al., 2013), it is important to investigate the molecular pathogenesis of the virus. Here, we show that six human NB cell lines, which behave similarly to a developing human fetal brain, can be infected with ZIKV. We also show that human differentiated NB neuronal cell lines (JFEN and T-268) are significantly resistant to ZIKV. This report illustrates the differential permissiveness of immature versus mature human neuron infections from a strain that is genetically similar to the ZIKV strain responsible for the epidemic in Brazil.

2. Material and Methods

2.1. Reagents and Cell Lines

Five NB cell lines were purchased from ATCC (New York, NY). CRL-2267, CCL-127, CRL-2271 (of male origin) CRL-2266, and CRL- 2149 (of female origin) were cultured in Eagle's Minimum Essential Media (EMEM) (ATCC, Manassas, VA) supplemented with 10% heat inactivated Fetal Bovine Serum (FBS), and L-Glutamine-penicillin-streptomycin solution (designated complete media) (Sigma, St. Louis, MO) at 37 °C, 5% CO_2. SMS-KCNR (of male origin) was purchased from Children's Oncology Group (Texas Tech University) and was cultured in RPMI-1640 (Sigma). Two terminally differentiated olfactory neuroblastoma cell lines, T-268 and JFEN, were a kind gift from Timothy J. Triche (Department of Pathology, Children's Hospital of Los Angeles, Los Angeles, CA 90027; 21). The stock cell cultures were grown in 25 mL or 75 mL flasks (Thermo-Scientific, Nunc, Rochester, NY). ZIKV strains PRVABC59 and MR 766 were provided by the CDC (kind gifts from Brandy Russel, Fort Collins, CO) and flavivirus envelop antibody (FE1), which cross-reacts with ZIKV, was purchased from Invitrogen (Cat. # MA1-71258). Two NB cell lines CRL-2266 and CRL-2267 were exposed to retinoic acid (RA) at 1 μM final concentration for 48 h (Breitman et al., 1980). Both cell lines exhibited partial differentiation as measured by morphologically and by immunostaining with anti-tubulin antibody.

2.2. Propagation of ZIKV

ZIKV strain (KU501215) was propagated in Vero cells (ATCC). The cells were grown in EMEM to 70% confluency in 25 mL tissue culture flasks (Nunc Inc., USA). Following removal of the culture media, virus inoculum was added to give a multiplicity of infection (MOI) of 0.1 to 0.05/cell or 1.0 to 1.5/cell. Flasks were incubated at 37 °C, 5% CO_2 with gentle agitation for 30 min. After incubation, 5 mL of complete media was added and the cells were maintained for 5–9 days or until cells exhibited cytopathic effects (CPE). Cells were trypsinized and cryopreserved in 50% FBS and 10% DMSO at −80 °C and used in subsequent studies.

2.3. Infection of Neuroblastoma Cell Lines With ZIKV

To examine the permissiveness of NB cell lines to ZIKV, all 6 undifferentiated cell lines and two olfactory NB cell lines, T-268 and JFEN (Sorensen et al., 1996), were grown in 25 mL tissue culture flasks to ~70% confluency, media was removed and immediately exposed to an estimated 0.1 to 0.05 MOI or 1.0 to 1.5 MOI in 1.0 mL serum free media. For maximum infectivity, the flasks were intermittently gently agitated for 1 h and then 5 mL of pre-warmed media was added. Control cultures were treated identically, except no ZIKV was added to the inocula. The cells were incubated at 37 °C, 5% CO_2. The cell cultures were observed every 12 h for CPE and images recorded on a digital microscope camera. Each experiment was repeated at least three times for low MOI and twice for high MOI infected cells.

2.4. Immunofluorescence Assay (IFA)

For immunocytochemical studies, each cell line was grown in 8-well chamber slides (Thermo Scientific, Nunc, Rochester, NY) with ~1×10^5 cells in 0.3 μL of media for 4–7 days. For seeding the NB cell lines, the stock cell cultures grown in the flasks were gently washed once with 1× PBS followed by trypsinization until single cell suspension and inactivation of trypsin with 1 mL FBS. The cells were counted on a hemocytometer chamber and adjusted to 1×10^5 cells/mL. The 8-well chamber slides were labeled, 100 μL of cells were then added to each well of the respective 8-well chamber slides, and 1 μL of fresh media was added containing 0.1–0.05 MOI of the virus. After 1 h of incubation, the cells were washed gently and 500 μL of fresh media was added. After 48 h culture, the media was removed from the slides and the cells were fixed by adding 800 μL of Streck Tissue Fixative (STF, Streck Laboratories Inc., LA Vista, NE), a non-ionic fixative, in each well and allowed to set overnight at 4 °C. The wells were then washed gently three times using 1× sterile Phosphate Buffered Saline (PBS) (Fisher Scientific, Fair Lawn, NJ) and then soaked with the blocking agent (containing 2% Bovine Serum Albumin (BSA) in 1× PBS) for 10 min. ZIKV was detected by utilizing mouse monoclonal antibody to flavivirus (Cat. # MA1-71258, Invitrogen). All antibody dilutions were carried out in 2% BSA in 1× PBS. The primary Ab was diluted to 1:10 in blocking buffer. In each chamber, 100 μL of diluted primary Ab was added to each well. The slides were incubated at 4 °C overnight in a humidified chamber and then washed three times in 1× PBS. Then, goat anti-mouse fluorescein conjugated secondary Ab was used at a working dilution of 1:40 with 100 μL in each well. The slides were incubated for 1 h at 37 °C in humidified chambers and then washed three times with PBS with 10 min incubation each time. The tops of the 8-well chamber were carefully removed without disturbing the fixed cells. The cells were washed in PBS three times and then mounted with glass coverslips, using a drop of mounting solution containing 50% glycerol, 50% PBS. The slides were observed at resolutions 10×, 40×, and 1000× (with oil) using an Olympus BX51 fluorescent microscope. Each experiment was repeated at least three times with low MOI and two times with high MOI of ZIKV.