Title: Dengue virus (DENV)-specific antibodies enhance Brazilian Zika virus (ZIKV) infection

Running title: ZIKV enhancement by dengue antibodies

Authors: Priscila MS Castanha, Department of Virology, Aggeu Magalhães Research Center, Oswaldo Cruz Foundation (FIOCRUZ), and Faculty of Medical Science/ Institute of Biological Science, University of Pernambuco, Recife, Brazil; Eduardo J. M. Nascimento, Center for Vaccine Research and Department of Infectious Disease and Microbiology, University of Pittsburgh, United States; Cynthia Braga, Aggeu Magalhães Research Center, Oswaldo Cruz Foundation (FIOCRUZ), Recife, Brazil, and Instituto de Medicina Integral Prof. Fernando Figueira, Recife, Brazil; Marli T. Cordeiro, Aggeu Magalhães Research Center, Oswaldo Cruz Foundation (FIOCRUZ), Recife, Brazil; Otávio V. de Carvalho, Aggeu Magalhães Research Center, Oswaldo Cruz Foundation (FIOCRUZ), Recife, and Universidade Federal de Viçosa, Minas Gerais, Brazil; Leila R de Mendonça, Elisa AN Azevedo, Rafael FO França, and Rafael Dhalia, Aggeu Magalhães Research Center, Oswaldo Cruz Foundation (FIOCRUZ), Recife, Brazil; Ernesto TA Marques Jr, Aggeu Magalhães Research Center, Oswaldo Cruz Foundation (FIOCRUZ), Recife, Brazil, and Center for Vaccine Research and Department of Infectious Disease and Microbiology, University of Pittsburgh, United States.

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Reprints or correspondence (post-publication): Dr. Ernesto TA Marques Jr., Center for Vaccine Research, 3501 5th Avenue, 9052 Biomedical Science Tower 3, Pittsburgh, PA 15261, USA. Phone: +1 (412) 648-2052; Fax: +1 (412) 624-4440; email: marques@pitt.edu.

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Abstract

Anti-Flavivirus antibodies are highly cross-reactive and may facilitate Zika virus (ZIKV) infection through the antibody-dependent enhancement (ADE) mechanism. We demonstrate that dengue-specific antibodies enhance the infection of a primary Brazilian ZIKV isolate in FcγRII-expressing K562 cell line. In addition, we demonstrate that serum samples from dengue-immune pregnant women enhanced ZIKV infection. These findings highlight the need of epidemiological studies and animal models to further confirm the role of ADE in the development of congenital and neurological complications associated with Zika virus infections.

Key words: Zika virus, antibody dependent enhancement, dengue antibodies
Background

Zika virus (ZIKV) is currently in the spotlight as a major public health concern worldwide [1]. Until recently, ZIKV infections in humans were associated with either asymptomatic or self-limiting exanthematic illness. However, the emergence of ZIKV in Polynesia and Brazil revealed the virus is associated with more severe clinical manifestations than previously reported, including neurological complications in adults (e.g. Guillain-Barré Syndrome) and microcephaly in newborns [2-4].

The factors responsible for the large variability of severe phenotypes [3] of ZIKV infection remain unclear. Dengue virus (DENV) antibodies have been shown to enhance ZIKV infection [5,6]. These antibodies cross-react with ZIKV structural proteins, facilitating virus uptake by cells expressing FcγR receptors through the mechanism of antibody-dependent enhancement (ADE) [5,6]. Due to the high rate of dengue transmission in Brazil, more than 90% of the adult population has been previously exposed to DENV, especially in the Northeast region [7]. Here, we used a panel of serum samples from individuals with different dengue immunity profiles to confirm that dengue antibodies enhance the infection of a Brazilian ZIKV isolate.

Materials and methods

Sera panel. We included well-characterized serum samples of (i) six laboratory-confirmed symptomatic dengue infections (primary and secondary dengue cases), (ii) twenty pregnant women with different dengue immunity profiles, and (iii) three dengue-naïve pregnant women.

Dengue symptomatic patient serum samples were collected from individuals enrolled in a cohort of dengue suspected cases conducted in the city of Recife [8], a large urban center
and hyperendemic area of dengue in Northeast Brazil. Sequential blood samples were collected from each patient during the acute (<7 days) and convalescent phases (10-15 days) as well as around 30 days after enrollment in the study. Additional samples were obtained 6 and 12 months later. Dengue cases were laboratory-confirmed by the combination of virus isolation in C6/36 cells and viral RNA detection by reverse-transcriptase polymerase chain reaction (RT-PCR) with dengue serology for IgM/ IgG by enzyme-linked immunosorbent assay (ELISA). The kinetics of IgM and IgG response were used to classify the cases as primary or secondary dengue infections. For this study, we included serum samples from primary (n=3) and secondary dengue cases (n=3) collected at the following time points: <7 days, 10-15 days, 30-40 days, and >260 days post infection [8].

Serum samples were obtained from healthy pregnant women included in a prospective dengue birth cohort study carried out in the same setting [9, 10]. These samples were collected between 2011-2012, and represent the dengue epidemiological profile of women at reproductive age shortly prior to the circulation of ZIKV in this area. Dengue serological profile of the mothers included in the cohort was determined by ELISA and plaque reduction neutralization test (PRNT) [9, 10]. In order to investigate ADE of ZIKV infection by DENV antibodies, two groups plus a controls group of mothers were selected based on their PRNT status: Group I, comprised serum samples from mothers with a monotypic DENV-immune profile (PRNT$_{50}$>20 to only one serotype) (n=10); Group II included sera from mothers with multitypic immunity (PRNT$_{50}$>20 to more than one DENV serotype) (n=10) (Table S2). Serum samples from DENV-naïve mothers enrolled in the cohort were also included in this study (n=3).
Samples were confirmed as DENV negatives by IgM/ IgG serology and PRNT. Details of the study design and data collection of both cohort studies have been previously described [8, 9]. A detailed description of the serological characterization of each sera included in this study have been described in the Supplementary Materials (Tables S1 and S2).

**Viruses.** ZIKV PE/243 and DENV-2 16681 (prototype strain) were used in the ADE assay. ZIKV PE/243 was isolated from a Zika case diagnosed in Pernambuco state, Northeast Brazil [11]. The virus strains were propagated in Vero cells, as described elsewhere [10]. The virus-containing supernatants were harvested from infected Vero cells and clarified by centrifugation (930g, 10 minutes, 4°C). Virus particles were precipitated with 50% polyethylene glycol (PEG 3100) in DMEM (Dulbecco’s modified Eagle medium). Briefly, the supernatant/ PEG mixture (proportion of 1:4) was incubated overnight at 4°C. After centrifugation (1,500g, 30 min., 4°C), the supernatant was discarded and the pellet containing the virus particles was resuspended in one hundredth of the original volume of supernatant/ PEG in DMEM with 25mM of HEPES. ZIKV PE/243 was titrated by plaque assay while DENV-2 16681 virus titer was determined by focus-forming assay [10].

**ADE assay.** FcγRII-expressing K562 cell line were exposed to ZIKV PE/243 in the presence of either a Flavivirus-naïve (AB human serum, MP, USA) or a dengue-immune serum sample (DENV-3 immune serum). ADE was determined by flow cytometry, as previously described [10]. ADE was measured as the n-fold increase in the percentage of virus-infected cells relative to that in a Flavivirus-naïve serum. DENV-2 16681 was used as a control for the assay.
Quantitative real-time PCR (qRT-PCR). ZIKV RNA was extracted from culture supernatants using QIAamp Viral RNA extraction kit (Qiagen Sciences, Valencia, CA, USA) following the manufacturer’s specifications. qRT-PCR was conducted by using the QuantiTect Probe RT-PCR Kit (QIAGEN, Valencia, CA, USA) with amplification in the Applied Biosystems 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA) following the manufacturer’s protocol. ZIKV primers (ENV1086F: 5’-CCGCTGCCCAACACAAG-3’ and ENV1162R: 5’-CCACTAACGTTCCTTTTGCAGACAT-3’) and probe (5’-VIC AGCCTACCTTGACAAGCTAGACACTCAA-BHQ1-3’) sequences for the quantitative virus detection assay were designed according to Lanciotti et al. (2008) [12]. The relative quantification of ZIKV RNA was assessed using the 7500 Software v2.0.6 (Applied Biosystems).

Ethical statement. The protocol was approved by the Ethical Committee of Aggeu Magalhaes Research Center (CAAE-0061.0.095.000-10) and of the Brazilian Ministry of Health (CONEP 25000.119007/2002-03; CEP68/02).

Statistical analysis. Student’s t test was used to compare percentage of infected cells and enhancing activity between two groups. The correlation between previous dengue immunity and ADE was determined using Spearman test. The level of significance was set at 0.05. Statistical analysis was performed using Graph Pad Prism, version 6.0e.
Results

We postulated that non-neutralizing cross-reactive dengue-specific antibodies enhance ZIKV infection of FcγR receptor-bearing phagocytes through the mechanism of ADE. In order to confirm that, FcγRII-expressing K562 cell line were exposed to the local Brazilian ZIKV isolate PE/243 in the presence of either a Flavivirus-naïve or a dengue-immune serum sample. ZIKV and DENV-2 infectivity were low on K562 cells infected without antibodies (1.23%±0.87 and 0.91%±0.39, respectively) or in the presence of Flavivirus-naïve serum (2.05%±1.09 and 2.11%±1.11, respectively). However, pre-incubation of ZIKV with a serum sample from a DENV-3 immune individual increased the infectivity of both viruses as compared to the Flavivirus-naïve sample (9.09%±5.58 for ZIKV and 3.45%±3.20 for DENV-2). ADE observed for ZIKV was three times greater than the one observed for DENV-2 (4.35±1.11 and 1.43±0.53, respectively; p=0.003). The experiment was independently performed four times in different days to assure reproducibility. A representative analysis is shown on Fig. 1A and B.

Next, we determined the kinetics of ADE of ZIKV infection in a panel of well-characterized serum samples from primary (n=3) and secondary (n=3) laboratory-confirmed dengue cases collected from acute (<7 days post onset of symptoms) and convalescent (10-15 days post onset of symptoms) phases up to after complete recovery (30-40 days and >260 days post onset of symptoms). ADE of ZIKV infection was not observed in samples collected during the febrile and acute phases (before serum conversion) in patients experiencing dengue primary infections (Fig. 2A). ADE was only observed in this group at later time points, after convalescence and recovery (Fig. 2A). In
contrast, serum samples from dengue secondary cases induced ADE of ZIKV infection regardless of the phase of infection analyzed (Fig. 2A).

Following, we selected a panel of serum samples from pregnant women immune to either DENV-3 alone (monotypic; n=10) or in combination with DENV-4 (multitypic; n=10), as determined by PRNT assays. All samples from DENV-immune mothers show ADE of ZIKV infection (20/20), whereas ADE was not observed among the DENV-naïve mothers (0/3). Serum samples from monotypic and multitypic dengue immune mothers induced greater ADE of ZIKV infection (16.41±3.12 and 17.08±2.86, for monotypic and multitypic, respectively; Fig. 2C) as compared to DENV-2 (1.54±0.62 and 0.76±0.36, for monotypic and multitypic, respectively; Fig. 2B). However, immunity to multiple DENV serotypes inhibited the infectivity of DENV-2 (p=0.003; Fig. 2B), whereas ADE of ZIKV was elevated in monotypic and multitypic groups (p=0.616; Fig. 2C).

ADE of ZIKV infection by dengue-specific antibodies was also observed after quantification of viral RNA, by qRT-PCR, in culture supernatants of infected K562 cells. Viral RNA levels increased up to 7-fold in the presence of dengue immune serum as compared to the Flavivirus-naïve serum (Fig. 2D). There was no difference in the ZIKV RNA levels collected from supernatants harvested from infected cells in the presence of dengue monotypic or multitypic maternal sera (p=0.1452).
Discussion

Although ZIKV has been linked to the increased incidence of congenital microcephaly cases [3, 4], the mechanisms underlying ZIKV transmission from mother to fetus remain unknown. Here, we demonstrated that the presence of dengue antibodies increased the infectivity of a primary Brazilian ZIKV isolate in human cell line expressing FcγRII receptors.

ADE of ZIKV infection in mononuclear phagocytes was first evidenced in the 80’s [13] and has been recently confirmed by others [5, 6]. Dejnirattisai et al. [5] showed that pooled convalescent serum and monoclonal antibodies (mAbs) derived from DENV-infected patients were able to promote ADE of ZIKV infection on the monocyte cell line U937 [5]. Our results confirmed these findings in a different cell line and also explored the kinetics of ADE on paired samples taken from the same subjects at different time points, contributing to better understand how pre-existing Flavivirus immunity influence ZIKV infection in vitro. We acknowledge that is not possible to definitively rule out previous ZIKV exposure of the patients included in our study, although there were no reports of ZIKV or microcephaly outbreaks in Brazil between 2004-2006 and 2011-2012, when the samples were collected. Of note, mAbs directed to the envelope dimer epitope (EDE) of DENV have been recently demonstrated to potently neutralize ZIKV infection and inhibit ADE in vitro [5, 14], pointing to a potential immunotherapy against ZIKV and opening the venues for the identification of ZIKV and DENV shared epitopes able to elicit neutralizing antibodies against both viruses [14].
Interestingly, we showed that sera from pregnant women - representative of the pregnant population of Recife, which was the epicenter of the microcephaly epidemic in Brazil – promote ADE of ZIKV infection \textit{in vitro}. Noteworthy, increased number of congenital disease associated to ZIKV infection has not been reported in Southeast Asia countries [15], where dengue has been hyperendemic for >5 decades and pregnant women are usually immune to all DENV serotypes. Unlike Asia, pregnant women from our setting are mostly immune to DENV-3 alone and probably have lower levels of DENV-associated antibodies than women exposed to several DENV infections [10]. Thus, we cannot exclude the possibility that background immunity and DENV-specific antibody levels of the population might contribute to increase disease severity of ZIKV infections. This probably explains the higher rates of congenital syndrome and neurological complications associated to ZIKV infections observed in Brazil as compared to Asian countries [4, 15], although virus virulence of the circulating ZIKV might also play a role.

In summary, we demonstrated that dengue-specific antibodies increase dramatically ZIKV infectivity \textit{in vitro} in phagocytes expressing FcγRII receptors. This finding might have implications to the immunopathogenesis of ZIKV infection in dengue-endemic areas. We acknowledge that the influence of ADE in determining severe disease \textit{in vivo} has been controversial in the dengue field [10]. To date, there has been no epidemiologic evidence of enhanced ZIKV illness during the ongoing epidemic in South and Central America. Although our data clearly demonstrate the ability of dengue antibodies to enhance ZIKV infection \textit{in vitro}, it is important to point out that the relevance of this mechanism \textit{in vivo} must be carefully explored. Additional
epidemiological and animal model studies are needed to elucidate the contribution of previous dengue immunity in mediating congenital and neurological complications associated with ZIKV infections.

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**Potential conflicts of interest.** All authors: No reported conflicts.
References


Figure legends

Figure 1. Antibody-dependent enhancement (ADE) of ZIKV infection by dengue-specific antibodies. FcγRII-expressing K562 cells were infected with ZIKV PE/243 in the absence of antibodies or in the presence of either a Flavivirus-naïve serum or a DENV-3 immune serum. ADE was measured as the n-fold increase in the percentage of virus-infected cells relative to that in a Flavivirus-naïve serum. (A) Percentage of DENV and ZIKV infected K562 cells in absence or in presence of serum. (B) ADE of ZIKV in K562 cells. ADE, Antibody-dependent enhancement; DENV, dengue virus; ZIKV, Zika virus.

Figure 2. Antibody-dependent enhancement (ADE) of ZIKV infection by dengue antibodies. (A) Longitudinal serum samples from dengue-infected patients were used to determine the kinetics of ADE of ZIKV infection after dengue exposure in individuals experiencing a primary (dotted lines) or secondary (solid lines) dengue infection. ADE of DENV (B) and ZIKV (C) was tested in the presence of a panel of serum samples from pregnant women with different dengue immune status, as determined by PRNT: monotypic (DENV-3) (n=10) and multitypic (DENV-3 and DENV-4) (n=10). (C) n-fold increase in the ZIKV vRNA quantification in the presence of monotypic or multitypic dengue immune serum relative to that in a Flavivirus-naïve serum. ADE, Antibody-dependent enhancement; DENV, dengue virus; ZIKV, Zika virus, vRNA, virus ribonucleic acid.
Figure 1
Figure 2

A

B

C

D

p = 0.03

p = 0.616

p = 0.1482