BRIEF REPORT

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Prior Dengue Virus Infection Is Associated With Increased Viral Load in Patients Infected With Dengue but Not Zika Virus

Gilberto A. Santiago,[1,a] Tyler M. Sharp,[1,2,a] Eli Rosenberg,[3] Iris I. Sosa Cardona,[1,4] Luisa Alvarado,[5,6] Gabriela Paz-Bailey,[1] and Jorge L. Muñoz-Jordán[1]

[1]Centers for Disease Control and Prevention, Dengue Branch, San Juan, Puerto Rico; [2]United States Public Health Service, Silver Springs, Maryland; [3]University at Albany School of Public Health, Department of Epidemiology and Biostatistics, Rensselaer, New York; [4]University of Puerto Rico, Department of Microbiology and Medical Zoology, San Juan; [5]Ponce Health Sciences University, Puerto Rico; [6]San Lucas Episcopal Hospital, Ponce, Puerto Rico

To evaluate potential enhancement of Zika virus (ZIKV) infection among patients with prior dengue virus (DENV) infection, we compared loads of viral RNA among patients infected with ZIKV (n = 1070), DENV-2 (n = 312), or DENV-3 (n = 260). Compared to patients without prior DENV infection, patients with prior DENV infection had significantly higher mean loads of viral RNA if infected with DENV-2 (10.6 vs 11.6 $\log_{10}$ GCE/mL, respectively; *t* test, *P* < .0001) or DENV-3 (10.3 vs 10.9 $\log_{10}$ GCE/mL; *P* < .0001), but not ZIKV (4.7 vs 4.7 $\log_{10}$ GCE/mL; *P* = .959). These findings provide evidence against in vivo enhancement of ZIKV by anti-DENV antibodies.

Key words: antibody-dependent enhancement; Puerto Rico; viral load; Zika virus.

INTRODUCTION

Following the emergence of dengue hemorrhagic fever (DHF) in Southeast Asia in the 1950s, antibody-dependent enhancement (ADE) was proposed as a pathophysiologic mechanism of increased disease severity [1]. Subsequent analyses demonstrated that ADE occurs when an antibody generated during an initial (primary) infection with 1 of the 4 dengue viruses (DENV-1–4) binds to virus during a subsequent (secondary) infection with a heterologous DENV; however, the antibody weakly neutralizes or fails to neutralize the infecting virus. Bound antibody then facilitates entry of DENV into Fc receptor-expressing immune cells (eg, macrophages, dendritic cells), resulting in more efficient virus uptake and replication and ultimately higher viral loads in serum. Studies suggest that high viral loads are associated with an imbalanced immune response and that these

factors together may result in increased vascular leakage and, consequently, DHF [1]. In line with this explanation, the magnitude of viremia during DENV infection has been associated with increased disease severity, and secondary infection has been associated with increased risk of developing DHF; however, magnitude of risk differs by a variety of factors, including time between and sequence of DENV infections, anti-DENV IgG antibody titer, and the infecting DENV [1].

Following the emergence of Zika virus (ZIKV) from the Pacific islands to the Americas and concurrent detection of unexpected clinical syndromes [2], various in vitro and animal studies reported evidence of ADE of ZIKV mediated by anti-DENV antibodies [3]. Findings include increased levels of ZIKV replication in vitro and production of pro-inflammatory cytokines and an increase in ZIKV pathogenesis in immunodeficient mice [3]. Conversely, ZIKV pathogenesis in nonhuman primates was unaffected by pre-existing anti-DENV antibodies, and it instead resulted in modulation of innate and cellular immune responses to ZIKV infection, blunted duration of viremia, decreased liver injury, and increased activity of cytotoxic T cells [4]. Therefore, data from humans is needed to evaluate potential ADE of ZIKV mediated by anti-DENV antibodies in vivo [5].

The objective of this analysis was to seek evidence for 1 indicator of ADE—viral RNA levels—by assessing if prior DENV infection was associated with increased load of viral RNA among patients symptomatically infected with DENV-2, DENV-3, or ZIKV.

METHODS

Serum specimens were collected from febrile patients presenting for care with acute febrile illness (AFI) identified by the following: (1) the Enhanced Dengue Surveillance System (EDSS), which operated in the emergency department of a secondary care hospital from 2005–2006 [6]; or (2) the Sentinel Enhanced Dengue Surveillance System (SEDSS), which has operated in the emergency department of 1 tertiary care hospital and 1 out-patient clinic since 2012 [7]. Both EDSS and SEDSS were approved by the Institutional Review Board at the Centers for Disease Control and Prevention. Written informed consent or assent was obtained from all study participants.

Specimens included in this analysis had been collected within 5 days of illness onset and tested positive by reverse transcription-polymerase chain reaction (RT-PCR) for infection with DENV-2 or DENV-3 during 2005–2006 or ZIKV during 2016. Load of viral RNA was quantified using a standard curve method based on RT-PCR assays to detect DENV or ZIKV nucleic acid [8, 9]. Briefly, the same primer set used to detect DENV or ZIKV was used to

generate virus-specific target amplicons, which were cloned individually into a TOPO TA vector system (Invitrogen, Carlsbad, CA). Insert-containing plasmids were expanded, purified, and used as templates to generate linear RNA transcripts by in vitro transcription (Lucigen, Middleton, WI). RNA transcripts were purified and the remaining DNA template was digested with Turbo *DNase*I (Invitrogen, Carlsbad, CA). Quality control and quantification of the linear RNA transcripts were performed by spectrophotometry and real time RT-PCR with and without reverse transcriptase to account for the presence of template DNA in the RNA solution. Serial dilutions with known concentrations of RNA transcript were performed for each target virus and tested with the corresponding RT-PCR assay. Load of viral RNA, expressed as genome copy equivalents per milliliter of specimen (GCE/mL), were determined by linear regression calculated from C_T values of each dilution and the corresponding RNA mass (converted to genome copies) detected per PCR reaction; this was further converted to copies per milliliter considering the RNA extraction procedure and the volume of specimen tested in each step of the process.

Status of primary versus secondary flavivirus infection (ie, serostatus) was defined by detection of anti-DENV immunoglobulin (Ig)G antibody by enzyme-linked immunosorbent assay (ELISA) [10]. Secondary infection was defined by detection of anti-DENV IgG antibody, whereas primary infection was defined by lack of detection of anti-DENV IgG antibody. DHF and severe dengue were defined according to the World Health Organization's 1997 and 2009 guidelines, respectively [11, 12].

We used SAS version 9.3 (Cary, North Carolina) to compare the distribution of participant demographics, DPO, and serostatus between patients infected with ZIKV, DENV-2, and DENV-3, conducting t and χ^2 tests as appropriate. For each virus, the mean $\log_{10}$ GCE/mL for patients with primary or secondary infection was computed with unadjusted comparisons made using t tests. To facilitate adjusted comparisons between virus and serostatus, a multivariable linear regression model of $\log_{10}$ GCE/mL was fit with predictors for days post-illness onset (DPO; treated as categorical), virus, serostatus, and their 2- and 3-way product interaction terms. Using a backward selection approach, terms with a P value < .05 were retained in the model. From this model, heterogeneity in differences in mean $\log_{10}$ GCE/mL by serostatus for each virus were estimated controlling for DPO.

RESULTS

We identified patients infected with ZIKV (n = 1070), DENV-2 (n = 312), and DENV-3 (n = 260) (Table 1). Patients with ZIKV infection were older and more frequently female than those infected with DENV-2 or DENV-3. The distribution of DPO of specimen collection was similar for patients infected with ZIKV, DENV-2, and DENV-3; however, no specimens collected on DPO 0 were available for patients infected with DENV-2 or DENV-3. The majority (>80%) of patients infected with DENV-2 or ZIKV were experiencing secondary flavivirus infection, whereas those infected with DENV-3 were more evenly distributed between primary and secondary infection.

Patients with ZIKV infection were hospitalized significantly less frequently than those infected with DENV-2 or DENV-3 (1.7% vs 50.6% and 43.8%, respectively; P < .0001) (Table 1). Patients with secondary infection with DENV-2 were hospitalized more frequently than those with primary infection (53.3%

Table 1. Characteristics of Patients Infected With ZIKV (n = 1070), DENV-2 (n = 312), or DENV-3 (n = 260), Puerto Rico, 2005–2006 and 2016

	ZIKV n = 1070	DENV-2 n = 312	DENV-3 n = 260
Participant characteristics			
Age in years, median (range)[a]	28 (0.08–88)	25 (0.5–81)	24 (0–84)
Female sex, n (%)[b]	623 (58.2)	127 (40.8)	129 (49.6)
Pregnant, n (%)	49 (4.6)	5 (1.6)	5 (1.9)
Hospitalized, n (%)	18 (1.7)	158 (50.6)	114 (43.8)
Day post-illness onset of specimen collection, n (%)			
0	89 (8.3)	0[c]	0[c]
1	332 (31.0)	60 (19.2)	55 (21.2)
2	294 (27.4)	63 (20.2)	77 (30.0)
3	245 (22.9)	84 (26.8)	62 (23.9)
4	110 (10.3)	66 (21.0)	39 (15.1)
5	0[c]	39 (12.4)	27 (10.4)
Serostatus			
Primary, n (%)	143 (13.4)	39 (12.4)	116 (44.8)
Secondary, n (%)	927 (86.6)	274 (87.8)	144 (55.4)

Abrreviations: DENV, dengue virus; ZIKV, Zika virus.

[a]Age was unavailable for 6 and 3 patients infected with DENV-2 and DENV-3, respectively.

[b]Sex was unavailable for 1 patient infected with DENV-2.

[c]Excluded from analysis.

vs 31.6%, respectively; $P = .01$). Frequency of hospitalization among patients infected with DENV-3 did not differ by status of primary versus secondary infection (39.6% vs 49.1%, respectively; $P = .13$). Only 1 patient met the case definition for DHF and had secondary infection with DENV-2. Among 16 patients who met the case definition for severe dengue, 5 had secondary and 0 had primary infection with DENV-2, and 7 had secondary and 4 had primary infection with DENV-3; these differences were not statistically significant.

Frequency of hospitalization was not different among patients with secondary versus primary infection with ZIKV (2.0% vs 1.4%, respectively; $P = .62$). Arthralgia was reported significantly more frequently among patients with secondary as compared to primary infection with ZIKV (82.3% vs 52.4%, respectively; $P < .01$); frequency of reported fever, rash, conjunctivitis, or all 4 symptoms was not significantly different by serostatus.

Load of viral RNA for each virus stratified by serostatus and DPO is shown in Figure 1A. Overall, load of viral RNA was significantly higher among patients with secondary compared to primary infection with DENV-2 (mean = 11.6 vs 10.6 $\log_{10}$ GCE/mL respectively [difference: +1.0]; $P < .0001$) or DENV-3 (mean = 10.9 vs 10.3 GCE/mL [difference: +0.6]; $P < .0001$), but not ZIKV (mean = 4.7 vs 4.7 $\log_{10}$ GCE/mL [difference: +0.0]; $P = .96$).

The multivariable model of $\log_{10}$ GCE/mL retained terms for DPO, virus, serostatus, and the interactions between virus and DPO ($P < .0001$), suggesting virus-specific differences in load of viral RNA over time. We observed interaction between virus and serostatus, indicating virus-specific differences in effect of

serostatus on load of viral RNA. These 2 factors together contributed nearly all of the observed variation in load of viral RNA ($r^2 = 0.92$). Although there was an overall significant interaction between virus and serostatus (Figure 1B), adjusted differences in load of viral RNA by serostatus were significant for DENV-2 (adjusted difference = +1.1; 95% CI, +0.7–1.5; $\log_{10}$ GCE/mL higher for secondary vs primary infection) and DENV-3 (+0.6; 95% CI, +0.3–0.8), but not ZIKV (+0.0; 95% CI, -0.2–+0.3). Similarly, the effect of serostatus on load of viral RNA was significantly different for DENV-2 and DENV-3 combined compared to ZIKV ($P < .0001$).

DISCUSSION

Utilization of 2 facility-based surveillance systems to identify patients with dengue or ZIKV disease enabled evaluation of load of viral RNA in humans as a proxy for ADE of ZIKV and DENV. After quantitation of load of viral RNA among patients experiencing primary or secondary infection with ZIKV, DENV-2, or DENV-3, we did not observe evidence to support in vivo enhancement of ZIKV mediated by anti-DENV antibodies; however, increased load of viral RNA was observed to enhance DENV-2 and DENV-3. Similarly, risk of hospitalization was higher among patients experiencing secondary infection with DENV-2, but not DENV-3 or ZIKV.

Evaluation of in vivo enhancement of ZIKV mediated by anti-DENV antibodies is challenging for several reasons. First, the large majority of pediatric and adult patients with ZIKV disease do not require in-patient care, among whom there is no apparent clinical phenotype by which to measure enhancement
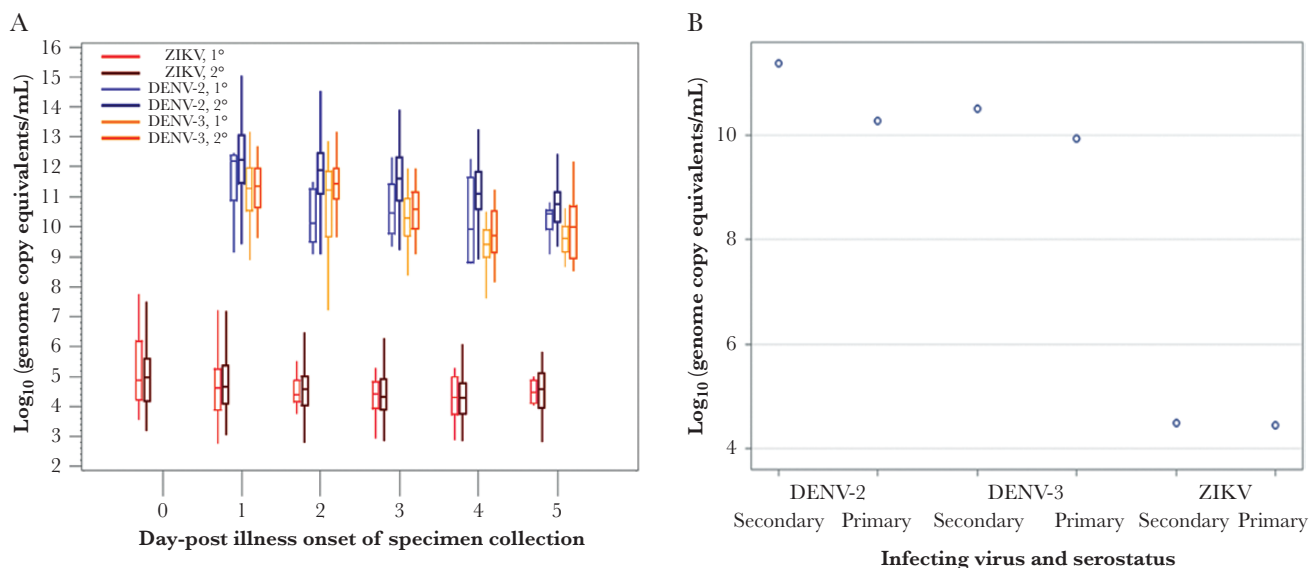
Figure 1. Load of Viral RNA in Patients Infected With ZIKV (n = 1070), DENV-2 (n = 312), or DENV-3 (n = 260), Puerto Rico, 2005–2006 and 2016 A, Viral load by serostatus and day post-illness onset of specimen collection. Upper and lower limits of plots indicate the maximum and minimum values, top and bottom limits of the boxes indicate the third and first quartiles, and the horizontal bar inside the box indicates the median. B, Median viral load by serostatus. DENV indicates dengue virus; ZIKV, Zika virus.

of acute ZIKV disease. Therefore, load of viral RNA or quantitation of cytokine levels may be the most appropriate in vivo markers of potential ADE. One study from Colombia utilized these measures to evaluate ADE among 45 patients infected with ZIKV and 20 infected with DENV-2 [13]. Although no association was observed between serostatus and load of viral RNA in patients infected with either ZIKV or DENV-2, interpretation of the findings was limited by a small sample size and lack of stratification of specimens by DPO, which is relevant as viremia decreases over time. Similar findings were observed in a case-control study of pregnant women with ZIKV infection in Brazil [14].

The magnitude of enhancement of DENV can differ according to the infecting DENV, as observed here, as well as the sequence of and time between DENV infections [1]. Consequently, we cannot rule out that enhancement of ZIKV may occur among other populations with a different history of prior infection with DENV. Moreover, even if anti-DENV antibodies do not increase Zika viral loads to cause severe disease, prior DENV infection may still be associated with the virulence or pathogenesis, or both, of ZIKV. For example, although there is no evidence to date to associate maternal serostatus with congenital Zika syndrome [15], initial findings suggest that having neutralizing antibodies against DENV-2 is associated with developing Guillain-Barré syndrome after ZIKV infection [16]. Therefore, further investigation of possible enhancement of ZIKV infection mediated by anti-DENV antibodies is merited, as findings are relevant to understanding the pathophysiology of severe complications of ZIKV infection as well as evaluation of ZIKV and DENV vaccines.

Though strengthened by evaluation of a robust number of patients infected with ZIKV, DENV-2, or DENV-3, this investigation still was subjected to limitations. First, different molecular diagnostic tests were used to screen patients with AFI for infection with DENV or ZIKV; however, because the performance of both tests is similar [8, 9], it is unlikely that individuals with low loads of viral RNA were missed. Second, although dengue patients experiencing secondary infection with DENV-2 were more often hospitalized than those with primary infection, hospitalization is an imperfect measure of disease severity due to variable clinical practices. Because of the small number of dengue patients that met the case definitions for DHF or severe dengue, we were unable to reliably assess the proportion of patients with severe disease by primary versus secondary infection with DENV-2 or -3. Last, in classifying patients with secondary flavivirus infection, we did not quantify anti-DENV IgG titers or identify the specific DENV(s) with which patients had been previously infected. Consequently, we were unable to determine if a specific range of anti-DENV IgG titer or sequence of past DENV infection may have been associated with differential increases in load of viral RNA.

In summary, our findings provide evidence against in vivo enhancement of ZIKV infection mediated by anti-DENV antibodies. Additional studies in other jurisdictions in populations with different histories of DENV infection prior to ZIKV infection should be conducted to further these findings.

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