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Enhancement of Zika virus pathogenesis by preexisting antilavivirus immunity

Susana V. Bardina^{1,*}, Paul Bunduc^{1,*}, Shashank Tripathi^{1,2,*}, James Duehr^{1,*}, Justin J. Frere¹, Julia A. Brown¹, Raffael Nachbagauer¹, Gregory A. Foster³, David Krysztof³, Domenico Tortorella¹, Susan L. Stramer³, Adolfo García-Sastre^{1,2,4,†}, Florian Krammer^{1,†}, and Jean K. Lim^{1,†}

¹Department of Microbiology, Icahn School of Medicine at Mount Sinai, New York, NY 10029, USA

²Global Health and Emerging Pathogens Institute, Icahn School of Medicine at Mount Sinai, New York, NY 10029, USA

³American Red Cross, Gaithersburg, MD 20877, USA

⁴Division of Infectious Diseases, Department of Medicine, Icahn School of Medicine at Mount Sinai, New York, NY 10029, USA

Abstract

Zika virus (ZIKV) is spreading rapidly into regions around the world where other flaviviruses, such as dengue virus (DENV) and West Nile virus (WNV), are endemic. Antibody-dependent enhancement has been implicated in more severe forms of flavivirus disease, but whether this also applies to ZIKV infection is unclear. Using convalescent plasma from DENV- and WNV-infected individuals, we found substantial enhancement of ZIKV infection in vitro that was mediated through immunoglobulin G engagement of Fcγ receptors. Administration of DENV- or WNV-convalescent plasma into ZIKV-susceptible mice resulted in increased morbidity—including fever, viremia, and viral loads in spinal cord and testes—and increased mortality. Antibody-dependent enhancement may explain the severe disease manifestations associated with recent ZIKV outbreaks and highlights the need to exert great caution when designing flavivirus vaccines.

Zika virus (ZIKV) is a mosquito-transmitted flavivirus endemic to parts of Africa and Asia. After its discovery in 1947, the virus remained relatively obscure until 2015, when a large outbreak occurred in Brazil and rapidly spread outward to other countries in South and Central America (1). Today, ZIKV is endemic to several U.S. territories, mainly Puerto Rico, and active ZIKV transmission has been reported in Florida and Texas as of February 2017

[†]Corresponding author. Email: adolfo.garcia-sastre@mssm.edu (A.G.-S.); florian.krammer@mssm.edu (F.K.); jean.lim@mssm.edu (J.K.L.).

*These authors contributed equally to this work.

SUPPLEMENTARY MATERIALS

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Materials and Methods

Figs. S1 to S12

Table S1

References (40–44)

(www.cdc.gov). The widespread outbreaks, the virus's association with microcephaly and other neurological disorders, and its long-term persistence in human tissues, leading to sexually mediated transmission and potentially infertility, have taken the medical community by surprise and raise major public health concerns. Whereas many questions remain unanswered about the unusual biology and disease spectrum of this flavivirus, there is urgency in determining whether antibodies against other flaviviruses could enhance ZIKV replication and disease pathogenesis. Antibody-dependent enhancement (ADE) of different dengue virus (DENV) serotypes has been shown to correlate with increased viremia and disease severity (2–4). ZIKV is phylogenetically related to other flaviviruses, such as DENV and West Nile virus (WNV), which cocirculate with ZIKV in many regions across the globe. In the United States and its territories, WNV and DENV are endemic, causing annual outbreaks of infection. South and Central America and Southeast Asia have experienced many outbreaks associated with all four DENV serotypes, which have resulted in hundreds of millions of DENV-seroconverted individuals in these geographical areas (5). Accordingly, as ZIKV continues to spread, understanding how preexisting flavivirus immunity affects ZIKV pathogenesis is a high priority.

In all flaviviruses, the envelope (E) protein is a primary target of neutralizing antibody responses (6, 7), and recent studies show that ZIKV E protein is highly structurally similar to that of DENV and WNV (8, 9). Several studies have evaluated DENV-specific monoclonal antibodies and/or a limited number of immune plasma samples for activity against ZIKV (10–14). Collectively, these studies showed that in vitro, some DENV antibodies are cross-reactive to ZIKV and can enhance ZIKV infection at specific concentrations. However, enhancement of ZIKV infection by DENV antibodies in vivo was not observed (12, 13). Another important question is whether enhancement can also be driven by immunity to other related flaviviruses, such as WNV. This is particularly pertinent given that more than 3 million people in the United States alone possess preexisting antibodies to WNV (15)—a virus that is also endemic throughout parts of Europe, Africa, the Middle East, and Australia (16).

To begin understanding the cross-reactivity of related flaviviruses to ZIKV and the implications for enhanced infection, we evaluated convalescent plasma from 141 immunoglobulin G (IgG)–positive DENV-infected blood donors and 146 IgG-positive WNV-infected blood donors identified through routine screening of blood donations in Puerto Rico and the United States, respectively (table S1) (17, 18). In contrast with the plasma of random blood donor controls in which no binding to ZIKV E protein was observed, a wide variability of binding activity to ZIKV E protein was detected for both DENV- and WNV-immune plasma (Fig. 1A). The former showed >350-fold greater binding ($P < 0.0001$) and the latter showed >35-fold greater binding ($P < 0.0001$) compared with controls. To evaluate the biological properties of these cross-reactive antibodies, we evaluated enhancement of ZIKV infection in vitro for each individual donor sample by using Fc γ receptor (Fc γ R)–bearing human-derived K562 cells. We observed a high level of overall ADE activity among the flavivirus-exposed individuals compared with controls ($P < 0.0001$; Fig. 1B), with significantly higher enhancement effects observed in DENV-exposed individuals ($P < 0.0001$). To further understand the relationship between ZIKV-reactive antibodies in human plasma and enhancement of infection, we plotted binding as a function

of enhancement for all DENV- and WNV-immune plasma. We found a strong positive correlation between levels of reactivity to ZIKV E protein and enhancement of infection for both the DENV- and WNV-immune plasma (Fig. 1, C and D). Because of the large number of study participants, ZIKV cross-reactivity and ADE among the DENV- and WNV-seropositive donors were evaluated for correlations to gender, age, severity of disease (fig. S1), and DENV serotype (fig. S2). Interestingly, for both DENV- and WNV-infected donors, a weak positive correlation to age was observed. DENV-1-positive samples had a trend toward higher enzyme-linked immunosorbent assay (ELISA) titers and ADE than DENV-4-positive samples, but the difference was not statistically significant (fig. S2). Evaluation of the ZIKV-neutralization potency of a subset of DENV- and WNV-immune plasma samples revealed that only the highly cross-reactive DENV-immune plasma samples were capable of neutralization (fig. S3). These data indicate that preexisting immunity to DENV and WNV can enhance ZIKV infection in vitro.

ADE is primarily mediated through the engagement of IgG antibodies with cell surface Fc γ Rs (19, 20). We therefore tested whether the ADE induced by DENV- and WNV-immune plasma was IgG-mediated. To do this, we assessed the ADE activity of plasma pooled from control, DENV-, and WNV-infected blood donors (15 per group) as compared with that in purified IgG from these same samples (hereafter, control, DENV, and WNV IgG). Blood donors used for pooling were individually tested for ADE activity (figs. S4 to S6). Of note, these plasma samples were tested for the presence of viral nonstructural protein 1 (NS1) and were found to be negative (DENV samples only; fig. S7). ZIKV was preincubated with serially diluted pooled plasma or purified IgG, and these mixtures were then used to infect K562 cells. Overall, enhancement activity was maintained for both DENV and WNV IgG, suggesting that IgG is the primary plasma component involved (Fig. 2A). Furthermore, IgG-depleted plasma did not enhance infection, indicating that ADE in K562 cells was solely attributable to the IgG fraction (Fig. 2B). Given the role of Fc γ Rs in mediating ADE, we also investigated the dependency of DENV- and WNV-specific ADE on IgG-Fc γ R engagement. To do this, we preincubated K562 cells in the presence or absence of purified Fc γ R binding inhibitor (BI; eBiosciences) before infection with ZIKV in the presence of serially diluted DENV, WNV, or control IgG. ZIKV ADE was ablated in the presence of the Fc γ R BI, suggesting that enhancement indeed occurs through Fc γ Rs (Fig. 2C). Because K562 cells mediate ADE through Fc γ RIIA (21–23), we next preincubated these cells either an antibody against CD32, which blocks IgG binding to Fc γ RII, or a control antibody against CD16, which blocks IgG binding to Fc γ RIII, a receptor that is not expressed on K562 cells. The addition of the antibody against CD32 completely inhibited the ADE induced by DENV and WNV IgG (Fig. 2D), whereas ADE was still robustly observed in the presence of the antibody against CD16 (Fig. 2E). This suggests that IgG-Fc γ RIIA interactions are specifically responsible for the enhanced infection in K562 cells. We further verified this by treatment of purified IgG from control, DENV-, or WNV-positive donors with peptide *N*-glycosidase (PNGase) F. PNGase F removes *N*-linked glycans from the Fc domain of IgG, which are necessary for Fc γ R engagement (24). This treatment specifically ablated the ability of DENV and WNV IgG to induce ADE (Fig. 2F), whereas the ability of purified IgG to bind ZIKV, as assessed by ELISA, was identical irrespective of PNGase F treatment (fig. S8). Together, our data demonstrate that IgG elicited by infection

with DENV and WNV is capable of mediating ADE of ZIKV through FcγR engagement in vitro.

Given the binding and enhancement effects observed with DENV- and WNV-convalescent plasma in vitro, we next evaluated whether ADE could occur during ZIKV infection in vivo. Several studies with numerous flaviviruses have shown that viral proteins antagonize the host innate immune response through targeting STAT2 (25–29). In pilot studies, we also evaluated the replication of ZIKV in different mouse strains and discovered that *Stat2*^{-/-}C57BL/6 mice displayed considerable morbidity and mortality in response to infection (30). Therefore, *Stat2*^{-/-} mice were given pooled immune plasma from control, DENV-, or WNV-positive donors. Two hours after transfer, all mice were infected with ZIKV strain PRVABC59 and monitored daily for survival, weight loss, and clinical symptom development.

In response to ZIKV infection, mice that received control plasma exhibited a 93.3% survival rate (Fig. 3A). In contrast, the vast majority of mice that received DENV-positive donor plasma succumbed to infection by day 8 (21.4% survival rate; $P < 0.05$ compared with mice receiving control plasma). These mice also exhibited significant weight loss (Fig. 3B) and an enhanced clinical symptom score (Fig. 3C), marked by the development of severe neurological symptoms including paralysis of several limbs and, in some cases, total body paralysis. We also observed a decreased survival rate among mice receiving WNV-immune plasma (60% survival; Fig. 3A), although this difference was not statistically distinct. Symptoms were less severe in mice receiving control plasma or phosphate-buffered saline (PBS). These results correlated directly with the differences in measured ZIKV cross-reactivity (Fig. 1).

To further assess clinical symptom development, core body temperatures were measured daily before and for several days after infection. Before infection, all mice showed an average body temperature between 36° and 37°C. Mice receiving control plasma showed a small but insignificant increase in body temperature on day 3, rising to an average of 37.6°C. However, mice that received DENV-positive plasma developed fever on day 3 postinfection, averaging 38.3°C. Mice receiving WNV-positive plasma also developed fever (38.0°C), although not as high as that in the DENV-positive plasma group (Fig. 3, D to G). To our knowledge, this is the first animal model showing a direct correlation between ADE and fever. Fever was the only symptom observed in the sentinel monkey in 1947 from which ZIKV was isolated and identified (31) and is the hallmark clinical feature in individuals with dengue hemorrhagic fever, West Nile fever, and ZIKV infections (1, 4, 32–34).

In humans, ADE caused by secondary infection with a heterotypic DENV strain can result in increased viremia, enhanced clinical symptoms, and mortality. To characterize ZIKV replication in the context of preexisting antibodies against DENV or WNV in vivo, we measured viral loads in the blood and several organs known to be associated with ZIKV infection and sexual transmission. In the blood of mice receiving control plasma or PBS, ZIKV RNA levels were elevated on day 3 and then decreased on day 6 postinfection (Fig. 4A). Mice receiving DENV-positive donor plasma had a significantly elevated viremia, with >10-fold higher levels on day 3 compared with mice receiving control plasma or PBS. Mice

that received WNV-immune plasma also had significantly higher viremia at this time point, with about a twofold increase compared with mice receiving control plasma or PBS. These differences in viremia were no longer detectable by day 6. Furthermore, ZIKV was detected in the spinal cord and testes in all groups of mice (Fig. 4, B and C), but titers were significantly elevated in mice receiving plasma from DENV- or WNV-seropositive donors. Immunofluorescent staining of ZIKV NS3 in the spinal cord and testes confirmed these observations, showing elevated ZIKV staining in mice receiving DENV- and WNV-infected donor plasma in comparison with that in the mice receiving control plasma or PBS (Fig. 4, D and E). Interestingly, ZIKV staining in the testes was primarily located in the region containing spermatid. We also tested ZIKV levels in several additional organs, including the brain, ovaries, and eyes, but found no differences in viral titers (Fig. 4, F to H). Together, our data suggest that ADE induced by cross-reactive DENV and WNV antibodies exacerbates *in vivo* viremia, which may lead to more efficient viral spread to the spinal cord and testes, two organs associated with human ZIKV disease and sexual transmission.

To further understand the relationship between DENV and WNV antibody titers and the potential for enhanced ZIKV disease *in vivo*, we evaluated a range of doses for control, DENV-, and WNV-immune plasma and their subsequent effects on ZIKV infection. High concentrations of DENV-immune plasma (200 μ l per mouse) resulted in protection against ZIKV infection, with 100% survival, no weight loss, and decreased symptoms. Lower concentrations (20 and 2 μ l per mouse) showed clear enhancement, with nearly identical survival, weight loss, and symptom development (fig. S9). In mice injected with WNV-immune plasma, we observed a dose-dependent response, with a trend of higher survival and better symptom outcome with higher concentrations of plasma. Notably, the highest dose of control plasma (200 μ l) resulted in a decrease in survival and greater symptom development, suggesting that a nonspecific response (e.g., from polyreactive antibodies) may occur at high plasma concentrations. However, comparison of control plasma-injected mice with mice receiving DENV- and WNV-immune plasma at high concentrations highlights the protective effects of cross-reactive flavivirus antibodies when present at sufficient concentrations. These data suggest that *in vivo* enhancement may occur optimally at low concentrations of ZIKV-reactive IgG, whereas high levels may be protective.

To evaluate which DENV-neutralizing titers correlate with ADE *in vivo*, we assessed the neutralizing activity of mouse plasma 2 hours after transfer of 200, 20, or 2 μ l of the pooled human plasma from controls or DENV-exposed individuals. We detected no neutralization activity in mice injected with 20 or 2 μ l of DENV-immune plasma and only residual neutralization of DENV in mice injected with 200 μ l of DENV-immune plasma (fig. S10). These results resemble titers of humans with waning immune responses after natural exposure to DENV (35, 36).

In addition to DENV and WNV, there are several clinically relevant flaviviruses and flavivirus vaccines that may also induce ADE. Particularly relevant in South America is yellow fever virus (YFV), for which a vaccine has been commercially available since the 1930s and has considerably reduced the disease burden (37). To test the extent to which preexisting immunity against YFV could promote ADE of ZIKV infection, serum samples from macaques vaccinated against YFV using the 17D strain were evaluated for binding to

ZIKV E protein and enhancement of ZIKV infection in vitro (fig. S11). Compared with prebleed samples, sera obtained from all three macaques 30 days after 17D vaccination (“early immune” phase) showed negligible reactivity to ZIKV E protein, as assessed by ELISA, and no ADE activity on K562 cells; weak reactivity and enhancement were observed for one of three macaques during the “late immune” phase (between 6 and 12 months after vaccination). For comparison, we also tested serum samples from DENV-infected macaques ($n = 4$), in which cross-reactivity to ZIKV E and in vitro ADE were robust at 30 days postinfection (early immune) and even stronger in the late immune phase. The response observed for DENV in the macaques fell well within the range observed in DENV-infected humans (Fig. 1). Although the numbers of animals used in this experiment are small, these data suggest that flaviviruses that are phylogenetically and serologically more distant from ZIKV may have progressively less cross-reactivity (38, 39). Another possibility is that attenuated flavivirus vaccines induce lower IgG titers that lead to less cross-reactivity and, therefore, less enhancement. In any case, our data suggest that YFV vaccination poses little risk of enhanced disease outcome if followed by ZIKV infection, particularly within the first 30 days after vaccination.

This study reports a large-scale analysis of ZIKV binding and enhancement by human immune plasma obtained from individuals infected with DENV and WNV. We show that the naturally occurring polyclonal antibody responses against WNV in humans are cross-reactive to ZIKV and can enhance ZIKV in vitro and in vivo. WNV-elicited antibodies appear to show less cross-reactivity and enhancement compared with DENV-elicited antibodies in vitro. Likewise, WNV-immune plasma in mice results in a less aggressive ADE phenotype compared with that in mice receiving the same amount of DENV-immune plasma. We also describe a mouse model of ZIKV infection that recapitulates ADE in vivo, resulting in clinically relevant phenotypes that mimic human disease. Furthermore, we show for the first time that fever in mice directly correlates with enhanced viral replication exacerbated by the presence of preexisting antibodies against other flaviviruses. Other studies evaluating the effect of DENV monoclonal antibodies on the pathogenesis of ZIKV in vivo found no enhancement of ZIKV disease (11, 12). Possible reasons for these differences may involve the mouse model used or the concentration of antibodies tested. Further, it is unclear whether strains of ZIKV from distinct lineages or geographic locations induce ADE to different extents. Preliminary studies using the Nigeria 1968 and Cambodia 2010 strains of ZIKV revealed distinct ADE curves in vitro (fig. S12). Furthermore, our study used polyclonal plasma for in vivo studies, rather than monoclonal antibodies, mimicking naturally occurring immunity. This may have also contributed to differences in outcome. Of note, it has been shown that antibodies against ZIKV can enhance DENV infection in vivo (11).

The breadth of flavivirus-induced ADE of ZIKV infection is unclear, but it includes at least DENV and WNV. Other clinically relevant viruses for which natural infection and large-scale vaccination campaigns have resulted in large numbers of seropositive individuals around the world—such as YFV, Japanese encephalitis virus, and tick-borne encephalitis virus—may be included. Given the high prevalence of DENV antibodies in the geographical areas most affected by ZIKV, our results suggest that preexisting immunity to DENV may have contributed to the rapid spread of ZIKV in the Americas and possibly is associated with

increased viremia and clinical symptoms, including microcephaly. In addition, the high prevalence of WNV antibodies in the United States raises concerns if ZIKV continues to spread into North America. Our results also have broad implications for vaccine efforts against DENV, WNV, and other flaviviruses. Cross-reactive antibodies induced by these vaccines might lead to enhanced infection when individuals are subsequently exposed to ZIKV. Our results highlight the urgent need for epidemiological studies in humans to understand the impact of preexisting flavivirus antibodies on ZIKV-induced disease and sequelae, while exercising great caution in the design and use of flavivirus vaccines in ZIKV-affected areas.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. ZIKV binding and enhancement of infection by DENV- and WNV-immune plasma

(A and B) Plasma samples from seropositive DENV-infected ($n = 141$), seropositive WNV-infected ($n = 146$), or seronegative control ($n = 15$) donors were evaluated for reactivity to ZIKV E protein by ELISA (A) or enhancement of ZIKV infection of K562 cells (B).

Calculations of area under the curve (AUC) based on serially diluted plasma measurements are shown. Black bars, geometric means. (C and D) Scatterplots showing the relationship between ZIKV binding and enhancement of ZIKV infection for DENV-immune (C) and WNV-immune (D) plasma. Each point represents one donor. Significance was analyzed by nonparametric unpaired Mann-Whitney U tests for (A) and (B) and by nonparametric Spearman's rank correlation for (C) and (D) (r , correlation coefficient). ** $P < 0.01$; **** $P < 0.0001$.

Fig. 2. Enhancement of ZIKV infection through IgG engagement of Fcγ receptors

(A) IgG purified from plasma pooled ($n = 15$ per group) from control, DENV-, or WNV-infected donors was evaluated for enhancement of ZIKV infection in K562 cells. (B) Pooled plasma samples from control, DENV-, or WNV-infected donors were evaluated for enhancement of ZIKV infection in K562 cells before and after IgG purification. (C to E) Purified IgG from control, DENV-, or WNV-seropositive donors was tested for enhancement of ZIKV infection in K562 cells in the presence or absence of Fcγ receptor binding inhibitor (FcγR BI) (C), antibodies (α) against CD32 (D), or antibodies against CD16 (E). (F) Plasma IgG from control, DENV-, or WNV-seropositive donors was incubated in the presence or absence of PNGase F before evaluation of ZIKV infection enhancement in K562 cells. The same control, DENV, and WNV IgG samples are shown in (A), (C), (D), and (E). All graphs show means $\pm$ SD.

Fig. 3. In vivo enhancement of ZIKV infection by DENV- and WNV-convalescent plasma

(A) 20 μ l of PBS or plasma from control, DENV-, or WNV-infected donors was administered intraperitoneally to *Stat2*^{-/-} mice 2 hours before intradermal inoculation with 5×10^3 plaque-forming units of ZIKV strain PRVABC59. The Kaplan-Meier survival curve is shown; significance was determined using the Mantel-Cox log-rank test and adjusted for multiple comparisons using the Bonferroni correction. Mice were monitored for (B) weight loss and (C) clinical score using a 6-point system ($n = 5$ per group), with a score of 7 awarded to deceased animals. Significance was determined using Student's *t* test and adjusted for multiple comparisons using the Bonferroni correction. In symptom score comparisons, the day with the highest average score per group was used. Daily body temperature measurements were taken from mice receiving PBS (D) or control (E), DENV- (F), or WNV-immune plasma (G). Statistically significant differences were calculated by comparing day 3 (the day of the highest total average temperature) and day 0 for each group. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. (B) and (C), means $\pm$ SEM; (D) to (G), means $\pm$ SD.

Fig. 4. In vivo ADE correlates with amplified ZIKV replication

(A) Blood viral titers of mice treated with PBS or control, DENV-, or WNV-positive donor plasma were assessed by real-time polymerase chain reaction (PCR) on days 3 and 6 postinfection. Viral titers were quantified by a plaque assay on spinal cords (B) and testes (C) isolated on day 6 postinfection from ZIKV-infected *Stat2*^{-/-} mice treated with PBS or a low dose of control, DENV-, or WNV-positive donor plasma. Paraffin-embedded spinal cords (D) and testes (E), taken on day 6 postinfection from ZIKV-infected *Stat2*^{-/-} mice treated with PBS or a low dose of control, DENV-, or WNV-positive donor plasma sections, were stained for ZIKV (green) and nuclear DAPI (4',6-diamidino-2-phenylindole; blue). Representative images are shown at 10× magnification; scale bars, 50 μm. Brains (F), ovaries (G), and eyes (H) were also evaluated for viral loads on day 6. **P* < 0.05; ***P* < 0.01. Means ± SEM. PFU, plaque-forming unit.