

# Influence of FcγRIIa-Expressing Cells on the Assessment of Neutralizing and Enhancing Serum Antibodies Elicited by a Live-Attenuated Tetravalent Dengue Vaccine

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**Background.** Recent trials of recombinant, live-attenuated chimeric yellow fever-dengue tetravalent dengue vaccine (CYD-TDV) demonstrated efficacy against symptomatic, virologically confirmed dengue disease with higher point estimates of efficacy toward dengue virus (DENV)3 and DENV4 and moderate levels toward DENV1 and DENV2. It is interesting to note that serotype-specific efficacy did not correlate with absolute neutralizing antibody (nAb) geometric mean titer (GMT) values measured in a Vero-based plaque reduction neutralization test assay. The absence of Fcγ receptors on Vero cells may explain this observation.

**Methods.** We performed parallel seroneutralization assays in Vero cells and CV-1 cells that express FcγRIIa (CV-1-Fc) to determine the neutralizing and enhancing capacity of serotype-specific DENV Abs present in CYD-TDV clinical trial sera.

**Results.** Enhancement of DENV infection was observed in CV-1-Fc cells in naturally exposed nonvaccine sera, mostly for DENV3 and DENV4, at high dilutions. The CYD-TDV-vaccinated sera showed similar enhancement patterns. The CV-1-Fc nAb GMT values were 2- to 9-fold lower than Vero for all serotypes in both naturally infected individuals and CYD-TDV-vaccinated subjects with and without previous dengue immunity. The relative (CV-1-Fc/Vero) GMT decrease for anti-DENV1 and anti-DENV2 responses was not greater than for the other serotypes.

**Conclusions.** In vitro neutralization assays utilizing FcγRIIa-expressing cells provide evidence that serotype-specific Ab enhancement may not be a primary factor in the serotype-specific efficacy differences exhibited in the CYD-TDV trials.

**Keywords.** antibody; dengue; enhancement; FcγR; vaccine.

The dengue viruses (DENV) exist as 4 serologically distinct, but antigenically related flaviviruses, DENV1–4, that are notable among mosquito-borne viral human pathogens for annually infecting an estimated 390

million people with 96 million clinically apparent cases [1]. Each of the 4 serotypes causes dengue fever (DF), and the more severe manifestation, dengue hemorrhagic fever (DHF), in proportions that change unpredictably over time and global location. Primary infection with a single DENV serotype (1) elicits both homotypic and heterotypic (cross-reactive) antibody (Ab) responses to the infecting serotype [2, 3], (2) is typically associated with mild DF cases, and (3) is considered to induce life-long immunity to the infecting serotype [4, 5]. However, immune responses induced against one serotype do not elicit long-term protection against infections by other serotypes, and these heterologous secondary infections are reported to be associated with the majority of cases of severe dengue, which nevertheless represent a low percentage of total cases [6–9].

Received 24 September 2015; accepted 3 November 2015.

Presented in part: Annual Meeting of the American Society of Tropical Medicine and Hygiene, New Orleans, LA; November 2–6, 2014 (Abstract 833).

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## Open Forum Infectious Diseases

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DOI: 10.1093/ofid/ofv172

Antibody-dependent enhancement (ADE) of DENV infection has been observed *in vitro* and has been hypothesized as a reason for the increase in severe cases upon secondary infection. Antibody-dependent enhancement is believed to occur when pre-existing nonneutralizing or poorly neutralizing anti-DENV Abs from a previous infection form DENV-Ab complexes with a newly infecting heterotypic serotype and infect cells via Fc $\gamma$  receptors [10–12]. Regardless of the mechanism of sensitization to severe dengue, a persuasive case can be made for the development of a tetravalent dengue vaccine with balanced responses against all 4 serotypes.

Recent efficacy trials of recombinant, live-attenuated chimeric yellow fever-dengue tetravalent dengue vaccine (CYD-TDV) showed protection against symptomatic, virologically confirmed dengue disease during the active-phase follow up (25 months postdose 1 [PD1]) [13, 14]. Serotype-specific point estimates of vaccine efficacy differed between DENV serotypes with high efficacy against DENV3 and DENV4 and moderate efficacy against DENV1 and DENV2. Although neutralizing Abs (nAbs), measured by Vero-based plaque reduction neutralization test (PRNT), were detected against all 4 serotypes, the respective geometric mean titer (GMT) for each serotype did not align with the level of efficacy for each serotype. Neutralization assay results with flaviviruses and other viruses suggest that assessment of the complex DENV nAb response may be greatly dependent on the cell substrate used in the procedure [15–20]. In this regard, key external opinion leaders in the dengue field highlighted the need for further investigation of the relationship between clinical efficacy levels and PRNT<sub>50</sub> titers in Vero cells, for instance by using alternative cells.

The primary sites of *in vivo* human DENV infection are Fc receptor-expressing cells of the monocyte/macrophage lineage [4]. Because the Vero cells typically used in DENV PRNT assays lack Fc receptors and the expression of Fc $\gamma$ R has been determined to be a crucial component of whether DENV-Ab immune complexes are infectious or neutralizing, we studied dengue neutralization in CV-1 cells transfected with Fc $\gamma$ R (CV-1-Fc) [21–26]. These CV-1-Fc cells were engineered to constitutively express transfected human Fc $\gamma$ RIIa (CD32A), the activating isoform of Fc $\gamma$ RII. The Fc $\gamma$ RIIa isoform leads to greater infectivity *in vitro* upon binding dengue immune complexes than Fc $\gamma$ RIIb (the inhibitory isoform) or Fc $\gamma$ RI and has been shown to be necessary for *in vitro* ADE of dengue infection [22, 24, 27, 28]. In addition, CV-1-Fc cells displayed differential sensitivity to dengue infection from non-Fc-bearing cells in sera from subjects infected with distinct DENV1 and DENV2 genotypes [29, 30].

One hypothesis, proposed by some experts to explain the lack of measurable protection against DENV2 in the phase 2b trial in Thailand (clinicaltrials.gov, NCT00842530), was that anti-DENV2-enhancing Abs formed all, or a large portion of, the anti-DENV2 response elicited by CYD-TDV [31]. Therefore,

we sought to assess neutralization in a cell line expressing Fc $\gamma$ R that was susceptible to infection via virus-Ab complexes to evaluate this possibility. We used parallel seroneutralization assays in Vero and CV-1-Fc cells to evaluate both neutralization and potential enhancement of the anti-DENV nAb response against all 4 dengue serotypes in plasma from a naturally dengue-infected cohort and sera from subjects vaccinated with 3 doses of CYD-TDV in both dengue-nonendemic and dengue-endemic regions. Assessment of the nAb response in subjects with previous dengue exposure is of importance because pre-existing immunity may impact both the quality and specificity and magnitude of responses induced by vaccination.

## MATERIALS AND METHODS

### Cells and Virus

Vero cells were used for PRNT assays. The CV-1-Fc cells transfected with Fc $\gamma$ RIIa (CV-1 CD32 R131 WT) and control-transfected CV-1c cells (CV-1 pcDNA5/FRT control), obtained from the laboratory of Dr. Jack Schlesinger (University of Rochester), were passaged in Eagle's minimal essential medium + 10% fetal bovine serum + 1% PenStrep [30]. Expression of Fc $\gamma$ RIIa in CV-1-Fc, CV-1c, and Vero cells was assessed by flow cytometry using CD32 monoclonal Ab (mAb) (clone 3D3; BD) every 2 weeks during passage on a BD Fortessa LSRII cytometer and analyzed using FlowJo (TreeStar). Dengue virus strains used in the PRNT assay were the 4 wild-type strains used to generate the CYD viruses in the tetravalent vaccine: DENV1 (PUO-359), DENV2 (PUO-218), DENV3 (PaH881/88), and DENV4 (1228). Viruses were grown in Vero cells at Sanofi Pasteur (VaxDesign).

### Naturally Infected Dengue Plasma Specimens

The Anti-Dengue Mixed Titer Performance Panel from SeraCare Life Sciences (catalog no. PVD201) is a 21-member panel of undiluted, unpreserved plasma from naturally infected individuals displaying a range of reactivities for antidengue immunoglobulin (Ig)M and IgG Abs determined by commercially available assays.

### Clinical Trial Sera

Sera samples from clinical trial subjects naive at baseline taken 28 days (postvaccine dose 3 [PD3]) after vaccination with CYD-TDV in a 0/6/12-month vaccination schedule were used from a study conducted in a nonendemic area for dengue ([www.clinicaltrials.gov](http://www.clinicaltrials.gov), NCT01134263) [32]. To address responses against DENV2, these samples were selected with a broad range of PD3 DEN2 Vero PRNT titers. Sera samples from clinical trial subjects preimmune at baseline before initial vaccination and 28 days after PD3 of CYD-TDV in a 0/6/12-month vaccination schedule were used from a study conducted in a dengue-endemic area ([www.clinicaltrials.gov](http://www.clinicaltrials.gov), NCT01187433) [33]. The sera

tested from this trial were paired prevaccination and PD3 samples that were selected to fit 4 profiles based on Vero prevaccination PRNT titer: seronegative, DENV2-dominant, DENV3-dominant, and multitypic (Supplementary Table 1). Use of clinical trial sera samples for these analyses was based on sufficient volume remaining and performed in accordance with study protocols for NCT01134263 and NCT01187433.

### Dengue Seroneutralization Assay

Concentrations of dengue nAb in Vero cells were measured by PRNT as published [34]. The CV-1-Fc cell PRNT assays were performed similarly. In brief, identical concentrations of virus for each serotype were used in parallel CV-1-Fc and Vero PRNTs and mixed 1:2 with 2-fold serial dilutions of sera for a final concentration range of 1:10 to 1:20480. Duplicate wells of each sample were evaluated when sufficient sera were available. After 6 days, cell monolayers were fixed and incubated with DENV serotype-specific mouse anti-envelope mAbs from Biotem (France): anti-DENV1 (D2-1F1-3), anti-DENV2 (3H5-1-12), anti-DENV3 (8A1-2F12), and anti-DENV4 (1H10-6-7), followed by incubation with alkaline phosphatase-conjugated goat antimouse IgG (Jackson ImmunoResearch). Plaques were visualized with SIGMAFAST BCIP/NBT alkaline phosphatase substrate in levamisole solution. Dengue virus plaques in CV-1-Fc cells for each serotype were typically smaller than plaques from parallel Vero assays but had the shape and density consistent with Vero DENV plaques (data not shown). The number of plaques was counted in high-resolution images of each well.

### Calculation of Neutralization Titers and Statistical Analysis

The PRNT<sub>50</sub> titer of the test serum sample is defined as the reciprocal of the serum concentration necessary to reduce the number of plaques by 50% compared to a serum-free virus only control. Plaque counts for all serial dilutions of serum are assessed to ensure a full dose response. The PRNT<sub>50</sub> titer is determined by linear regression analysis of the log<sub>10</sub>-transformed plaque counts from 4 selected points that span the 50% neutralization cutoff. All PRNT titers are PRNT<sub>50</sub> unless otherwise specified. The PRNT<sub>50</sub> values of <10 are given a value of 5 for calculation of GMT values. The CV-1-Fc/Vero GMT ratio (GMTR) for a particular subset was determined by dividing the CV-1-Fc PRNT<sub>50</sub> titer by the Vero PRNT<sub>50</sub> titer for each individual sample and calculating the geometric mean of all individual CV-1-Fc/Vero ratios. Comparisons of sera neutralization titers in Vero and CV-1-Fc cells were performed using a Mann–Whitney *U* test (GraphPad Prism).

## RESULTS

### FcγRIIa Is Stably Expressed in CV-1-Fc Cells

The expression of FcγRIIa (CD32) was periodically monitored by flow cytometry on CV-1-Fc cells, CV-1 cells transfected with an empty vector (CV-1c), or Vero cells. Expression of CD32 is

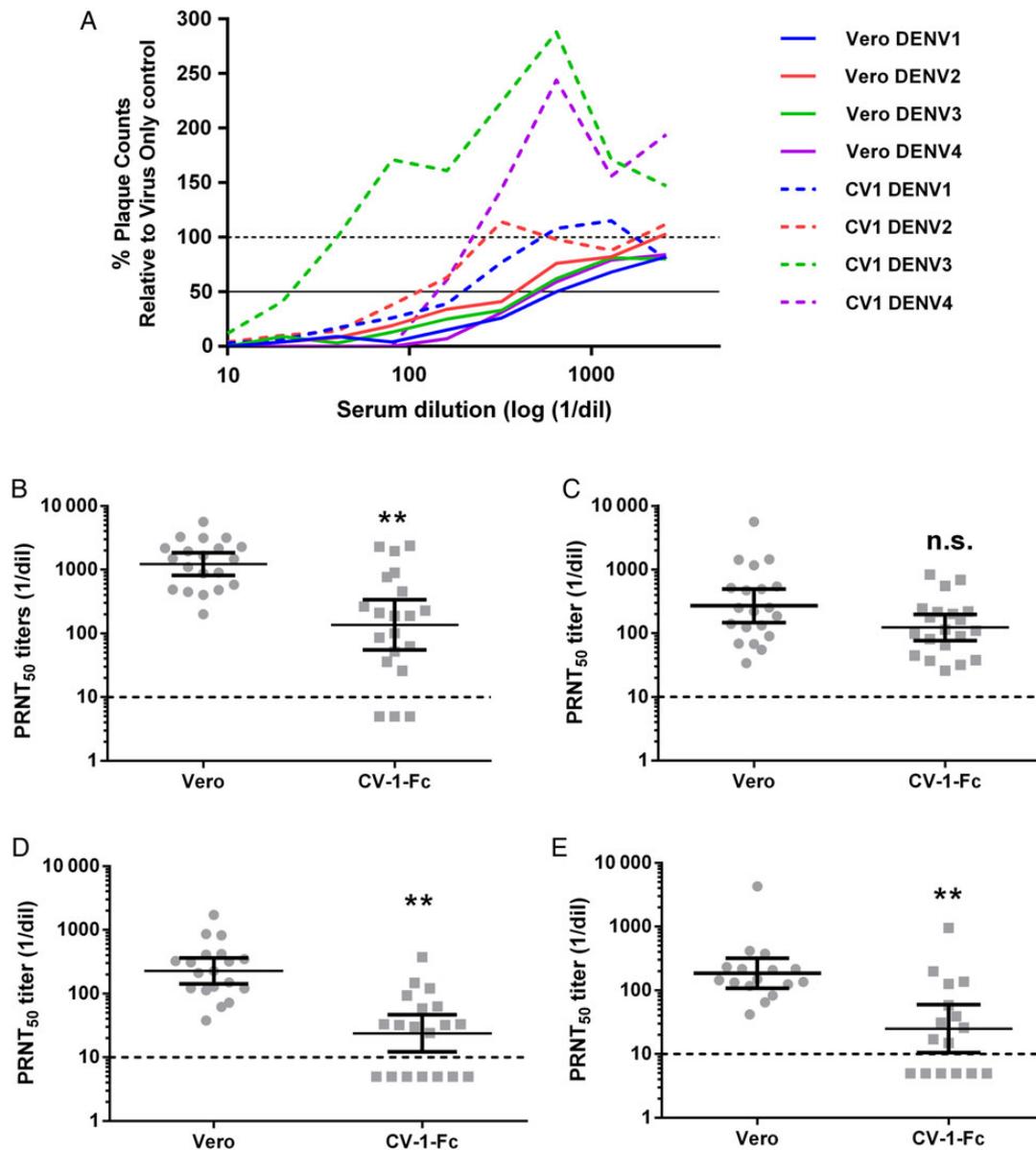
shown to be approximately 2 logs greater in CV-1-Fc cells than CV-1c and Vero cells over a 2-month period. In the day 74 panel, 2 different groups of CV-1 cells that had been thawed from the same parent lot at different times and maintained for different numbers of passages display the same level of FcγRIIa expression, suggesting that expression of this molecule is stable in this cell line (Supplementary Figure 1).

### The Capacity for Enhancement Is Observed in CV-1-Fc Cells Mostly for Dengue Virus (DENV)3 and DENV4

The FcγRIIa receptor has been previously shown to be linked with in vitro dengue ADE responses in a flow cytometry-based K562 model [35]. In our study, we assessed enhancement of virus infection in the CV-1-Fc and Vero PRNT cells relative to the virus-only control run concurrently with each experiment. We arbitrarily defined enhancement as  $\geq 150\%$  of the plaque count of the virus-only control well for a given serotype. Enhancement of dengue infection was observed in CV-1-Fc cells for a large proportion of subjects at dilute sera concentrations greater than the 50% and 100% neutralization thresholds in each of the cohorts assessed: (1) the naturally infected panel, for which the infecting dengue serotype is not known, and (2) the CYD-TDV clinical trial sera in dengue-naïve and dengue-endemic regions. A representative neutralization profile for a single subject is displayed in Figure 1A. As expected for an FcγR-negative cell line, enhancement was not observed in the parallel Vero PRNT assays for any subjects in the dengue-naïve cohort and in a very small number in the naturally infected cohort. Comparison of the naturally infected and clinical trial cohorts in CV-1-Fc cells showed that there were fewer instances of enhancement in the vaccinated subjects from the dengue-naïve cohort (27%) than in the naturally infected (53%) or the dengue preimmune (50%) cohort. It is interesting to note that the capacity for enhancement by sera in CV-1-Fc cells occurred more frequently for DENV3 and DENV4 than for DENV1 or DENV2 with similar serotype-specific trends between naturally infected and vaccinated samples (Table 1). In fact, enhancement of dengue infection in the presence of CV-1-Fc cells was not detected for DENV2 in sera from CYD-TDV vaccinees in either clinical trial.

### Plaque Reduction Neutralization Test<sub>50</sub> Titers Assessed in CV-1-Fc Were Lower Than in Vero for All 4 Serotypes: The Dengue Virus 2 Antibody Response Was not More Enhancing Than the Other 3 Serotypes

The CV-1-Fc and Vero PRNT assays were performed on samples from the naturally infected dengue cohort with unknown dengue exposure history. A subset of the samples tested in the CV-1c cells that did not express FcγRIIa displayed PRNT<sub>50</sub> titers that were similar to or higher than the corresponding Vero titers (data not shown). Samples from the naturally infected cohort had PRNT<sub>50</sub> GMT values for CV-1-Fc cells (DENV1,



**Figure 1.** CV-1-Fc cells are capable of in vitro enhancement; CV-1-Fc plaque reduction neutralization test (PRNT)<sub>50</sub> titers are lower than Vero PRNT<sub>50</sub> titers in naturally infected dengue samples. (A) A representative profile of the enhancement of dengue viruses (DENV) infection at very dilute sera conditions in CV-1-Fc cells in a single donor. Percentage of plaque counts relative to virus-only control is defined as the number of plaques generated in a PRNT by a dengue virus-sera mixture divided by a dengue virus-only mixture at specific dilutions over a dilution range of sera for the indicated dengue virus serotype in the indicated cell lines. Plaques were visualized by immunostaining with serotype-specific E monoclonal antibodies. Solid line and dotted line represent 50% neutralization and 100% neutralization, respectively. (B–E) PRNT<sub>50</sub> titers (1/dilution) are displayed for each individual plasma sample tested against (A), DENV1; (B), DENV2; (C), DENV3; (D), DENV4 are displayed for Vero, filled circle, and CV-1-Fc, filled square, cells. The solid black lines and error bars represent the geometric mean titer value and the 95% confidence interval, respectively. One dengue-negative placebo sample was removed from this analysis. The dotted line at PRNT<sub>50</sub> titer value of 10 represents the lower limit of quantitation. Statistical comparison of Vero and CV-1-Fc for each serotype was assessed using a Mann-Whitney *U* test; \*\**P* < .01. n.s., not significant.

136.5; DENV2, 123.4; DENV3, 23.8; DENV4, 25.0) that were lower than the corresponding values in Vero cells (DENV1, 1229; DENV2, 270.5; DENV3, 227.5; DENV4, 185.2) for each of the 4 serotypes (Figure 1B–E), and the relative difference between CV-1-Fc and Vero GMT for DENV2 was smaller than the other 3 serotypes.

Next, we assessed neutralizing titers in sera from vaccinated subjects who were seronegative at baseline. We examined PD3 sera from clinical trial subjects in a nondengue-endemic region that received 3 doses of CYD-TDV. Similar to the naturally infected cohort, these sera samples displayed a decrease in CV-1-Fc PRNT<sub>50</sub> GMT values (DENV1, 65.5; DENV2, 72.3; DENV3,

**Table 1. The Number of Subjects per Serotype in Each Cohort That Displayed Enhancement of DENV Infection in the Indicated Cell Type**

Serotype	Vero	Vero	CV-1-Fc	CV-1-Fc	CV-1-Fc
	Natural Infection	Dengue-Naive; CYD-TDV	Natural Infection	Dengue-Naive; CYD-TDV	Dengue-Immune; CYD-TDV
DENV1	0/21 <sup>a</sup>	0/29	9/21	6/29	6/20
DENV2	1/21	0/29	5/21	0/29	0/20
DENV3	5/21	0/29	20/21	16/29	18/20
DENV4	1/21	0/29	11/21	9/29	16/20
Total	7/84 (8%)	0/116 (0%)	44/84 (53%)	31/116 (27%)	40/80 (50%)

Abbreviations: CYD-TDV, chimeric yellow fever-dengue tetravalent dengue vaccine; DENV, dengue virus.

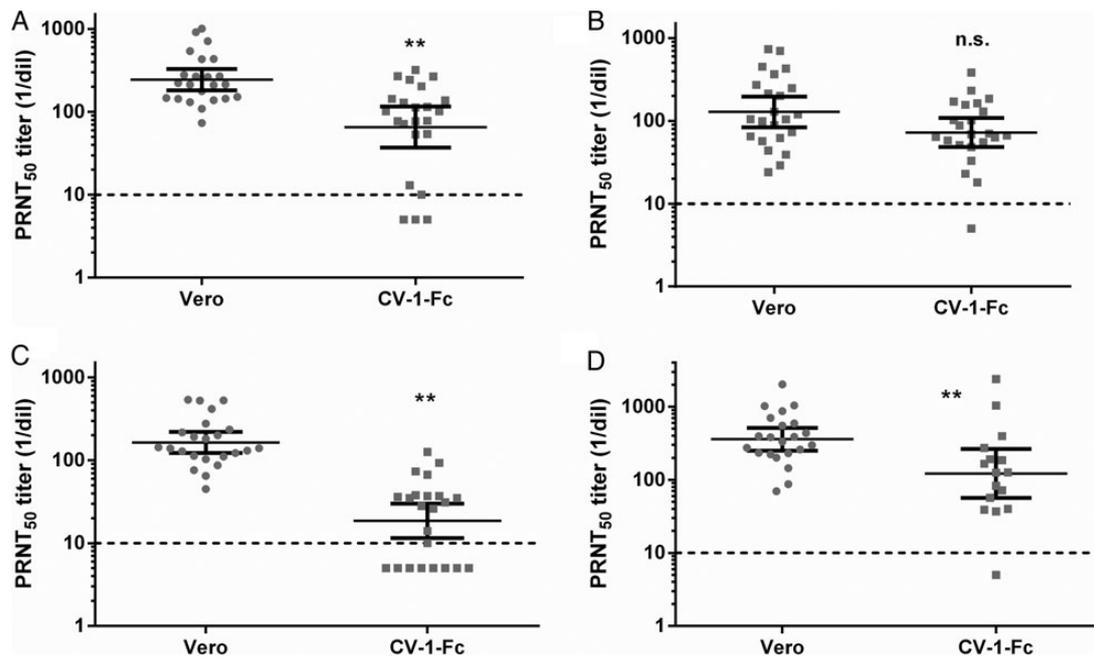
<sup>a</sup> Enhancement is defined as at least 1 serum-virus dilution with plaque counts  $\geq 150\%$  of the virus-only control well for a given serotype.

18.6; DENV4, 122.3) compared with Vero values (DENV1, 244.7; DENV2, 128.3; DENV3, 163.8; DENV4, 359.7) across all 4 serotypes (Figure 2), and the relative difference between CV-1-Fc and Vero GMT for DENV2 was smaller than the other 3 serotypes. In addition, the serotype hierarchy of the CV-1-Fc/Vero relative difference was the same for both groups (DENV3>DENV4>DENV1>>DENV2). This suggests that natural infection and CYD-TDV vaccination in dengue-naive individuals elicited similar nAb profiles in CV-1-Fc cells. To ensure that the assessment of the neutralizing capacity of anti-DENV2 Abs in CV-1-Fc cells was not greatly affected by the amount of

nAb present, the dengue-naive clinical trial sera samples were delineated by Vero DENV2 PRNT<sub>50</sub> titer into low (0–40), moderate (40–200), and high (>200) groups. In all 3 of these groups, the DENV2 CV-1-Fc/Vero GMTR was the smallest of the 4 serotypes (data not shown).

**Previous Dengue Immunity Does not Lead to More Enhancing Dengue Virus 2 Antibody Responses**

Because of the lack of correlation between efficacy and Vero PRNT<sub>50</sub> GMT values in the phase 2b clinical trial in a dengue-endemic region of Thailand, and because severe dengue



**Figure 2.** CV-1-Fc plaque reduction neutralization test (PRNT)<sub>50</sub> titers are lower than Vero PRNT<sub>50</sub> titers in recombinant, live-attenuated chimeric yellow fever-dengue tetravalent dengue vaccine (CYD-TDV) subjects that were dengue-naive at baseline. The PRNT<sub>50</sub> titers (1/dilution) are displayed for each individual sera sample tested against (A), dengue virus (DENV)1; (B), DENV2; (C), DENV3; (D), DENV4 are displayed for Vero (filled circle) and CV-1-Fc (filled square) cells. The solid black lines and error bars represent the GMT value and the 95% confidence interval, respectively. Subjects that received placebo vaccinations were removed from this analysis. The dotted line at PRNT<sub>50</sub> titer value of 10 represents the lower limit of quantitation. Statistical comparison of Vero and CV-1-Fc for each serotype was assessed using a Mann–Whitney *U* test; \*\**P* < .01. n.s., not significant.

**Table 2. CYD-TDV Vaccination of Previously Immune Subjects Did not Induce Anti-DENV2 Abs That Were More Enhancing Than the Other 3 DENV Serotypes<sup>†</sup>**

Serotype	Prevaccination CV-1-Fc PRNT <sub>50</sub> GMT (95% CI)	Prevaccination Vero PRNT <sub>50</sub> GMT (95% CI)	Prevaccination CV-1-Fc/Vero GMTR	Postdose 3 CV-1-Fc PRNT <sub>50</sub> GMT (95% CI)	Postdose 3 Vero PRNT <sub>50</sub> GMT (95% CI)	Postdose 3 CV-1-Fc/Vero GMTR
DENV1	72 (44, 119)	85 (41, 177)	0.75	348 (205, 591)	904 (449, 1822)*	0.39
DENV2	115 (82, 162)	202 (120, 339)	0.57	650 (422, 999)	2233 (1273, 3914)*	0.29
DENV3	66 (25, 171)	217 (61, 766)	0.30	393 (200, 771)	1624 (729, 3618)*	0.24
DENV4	24 (14, 41)	26 (14, 49)	0.91	202 (146, 278)	833 (416, 1667)*	0.24

Abbreviations: Abs, antibodies; CI, confidence interval; DENV, dengue virus; GMT, geometric mean titer; GMTR, geometric mean titer ratio; PRNT, plaque reduction neutralizing titer.

<sup>†</sup>Samples that were naive at baseline before vaccination were removed from the analysis for both groups.

\**P* < .05.

cases often occur upon secondary infection, we investigated how the presence of prior dengue immunity affected the neutralizing capacity of anti-DENV Abs induced by CYD-TDV vaccination in CV-1-Fc and Vero PRNTs with sera from a clinical trial in a dengue-endemic region.

In the prevaccination samples from the dengue-endemic region, the CV-1-Fc/Vero GMTR displayed less than 1.5-fold decreases for DENV1 and DENV4 and 2- to 3-fold decreases for DENV2 and DENV3 (Table 2, prevaccination). The PD3 samples from the dengue-endemic region had CV-1-Fc PRNT GMT values 2.3- to 4.5-fold lower than Vero values for all 4 DENV serotypes (Table 2, Postdose 3), similar to data in vaccinees from the dengue-nonendemic region (Figure 2). In these samples, it appears that 3 doses of CYD-TDV vaccination in previously immune volunteers may induce a further overall decrease in the CV-1-Fc/Vero GMTR compared with prevaccination samples regardless of serotype.

To specifically compare the effect of dengue preimmunity on the CV-1-Fc/Vero GMTR, the dengue-endemic PD3 samples that were naive at baseline were excluded from the serotype-

specific analysis. For the PD3 samples, the CV-1-Fc values for DENV2 did not show a greater difference relative to Vero than the other 3 serotypes. Consistent with our previous data, this suggests that prior dengue exposure does not contribute to induction of enhancing anti-CYD2 Abs relative to the other 3 DENV serotypes (Table 2, Postdose 3). Whether the nature of the prior dengue immunity was DENV2-dominant, DENV3-dominant, or multitypic did not greatly alter the serotype hierarchy GMTR trends displayed for all dengue-immune samples (data not shown).

## DISCUSSION

In this study, we have compared nAb responses against each of the 4 DENV serotypes in the sera of vaccinated subjects via PRNTs incorporating cells that do or do not express the FcγRIIIa receptor. Our observations that CYD-TDV vaccination did not display in vitro enhancement against DENV2 (measured by CV-1-Fc/Vero GMTR) relative to the other 3 serotypes in either dengue-naive or dengue-immune subjects (Table 3) imply that

**Table 3. The CV-1-Fc/Vero Geometric Mean Titer Ratio for Each of the 4 DENV Serotypes in the 3 Dengue-Positive Cohorts<sup>a</sup>**

Cohort	Naturally Infected <sup>b</sup> GMTR (95% CI) n = 20	Dengue-Naive; CYD-TDV Vaccination <sup>c</sup> GMTR (95% CI) n = 23	Dengue-Immune; CYD-TDV Vaccination <sup>c</sup> GMTR (95% CI) n = 16
Serotype			
DENV1	0.11 (.06, .23)	0.27 (.16, .45)	0.39 (.26, .56)
DENV2	0.46 (.32, .65)	0.56 (.39, .80)	0.29 (.17, .51)
DENV3	0.11 (.05, .21)	0.11 (.07, .18)	0.24 (.17, .34)
DENV4	0.14 (.08, .24)	0.29 (.16, .57)	0.24 (.13, .44)

Abbreviations: CI, confidence interval; CYD-TDV, chimeric yellow fever-dengue tetravalent dengue vaccine; DENV, dengue virus; GMTR, geometric mean titer ratio.

<sup>a</sup> Samples that were not infected with dengue (naturally infected and dengue-naive vaccinated) or naive at baseline before vaccination (dengue-immune vaccinated) were removed from analysis for both groups.

<sup>b</sup> Samples were from an unknown time after natural dengue infection.

<sup>c</sup> Samples were from 28 days after third vaccination dose.

lesser levels of efficacy toward DENV2 in CYD-TDV clinical trial may not be related to enhancement. If the CYD2 component of the CYD-TDV vaccine had induced higher levels of anti-DENV2 Abs capable of enhancing dengue infection compared with the other serotypes, the opposite outcome—ie, a greater decrease in CV-1-Fc GMT relative to Vero—would have been expected for anti-DENV2 responses. Indeed, comparison of the naturally infected cohort and the CYD-TDV-vaccinated dengue-naive subjects suggests that CYD-TDV vaccination does not induce anti-DENV2 Ab responses that are more enhancing than wild-type infection.

Sera from CYD-TDV clinical trial subjects in both dengue-endemic and nonendemic regions exhibited neutralizing activity for all 4 serotypes in CV-1-Fc cells at the least dilute sera concentration (1:10) that most closely resembles the undiluted condition of sera in the body. Because the capacity for enhanced viral replication in CV-1-Fc cells in vitro occurred only at more highly diluted sera concentrations (between 320- and 1280-fold depending on serotype), the likelihood of immune enhancement in vivo from CYD-TDV appears unlikely. Enhancement of DENV infection relative to the virus-only control at these highly dilute sera concentrations occurred frequently toward DENV3 and DENV4 Abs, whereas little enhancement was detected toward DENV1, and no enhancement was noted toward DENV2. In addition, we did not observe a relationship between the magnitude of the nAb titer and enhancement in any of the cohorts studied (data not shown). These CV-1-Fc neutralization titers suggest that the lower level of efficacy for DENV serotypes 1 and 2 in the recent phase 2b and phase 3 clinical trials is unlikely to be affected by enhancing Abs against these serotypes generated by CYD-TDV vaccination, should such an in vitro assay be predictive of any clinical significance. Moreover, the absence of in vitro enhancement toward DENV2 in CYD-TDV vaccines is consistent with the small number of severe dengue cases attributed to DENV2 in the phase 2b efficacy trial despite lower levels of efficacy against this serotype [13, 14, 31].

These observations showing no particular in vitro enhancement against DENV1 and DENV2 are also consistent with the fact that protection against severe dengue disease was observed against all 4 serotypes during the active phase follow up (25 months PD1) of the phase 3 efficacy trials conducted in more than 20 000 vaccinated subjects in Asia-Pacific (NCT01373281) and Latin America (NCT01374516). The vaccine elicited overall significant reduction of symptomatic dengue disease cases and, in both trials, exhibited an efficacy against DHF and clinically important reductions in dengue-related hospitalizations during the active phase follow up [13, 14]. Another point to consider is the nature and quality of the Abs examined and their relationship with the Fcγ receptors involved in these investigations. FcγRIIIa preferentially binds complexed IgG Abs with a much lower affinity than FcγRIa, which binds both monomeric and complexed forms of IgG [22, 28]. In this FcγRIIIa-CV-1 system, Abs that

bind with high affinity on multiple viral epitopes may have a greater chance to bind FcγRIIIa and, therefore, enhance infection, whereas Abs with lower affinity that are less likely to form viral complexes may bind to a lesser extent and are less enhancing. Further investigation in this manner should examine (1) the impact of the homotypic and heterotypic characteristics of anti-DENV nAbs and (2) whether low-affinity Abs are typically more enhancing than high-affinity Abs when tested by similar in vitro assays. Analysis of the neutralizing capacity of homotypic and cross-reactive Ab generated in CV-1-Fc cells via statistical modeling [36], or measurement of Ab affinity [37], is beyond the scope of the current study, but knowledge of the homotypic and heterotypic Ab profile elicited by CYD-TDV in the dengue-naive and dengue-immune individuals compared with the Abs elicited by natural infection may be crucial in understanding the putative efficacy of vaccination-induced Abs.

This study presents some limitations, because neutralization assays utilizing an FcγRIIIa-transfected monkey kidney-derived cell line are limited in their capacity to mimic the physiological coexpression of both FcγRIIIa and FcγRIIb isoforms found in most human dengue target cells. Recent work has shown that FcγRIIIa via its immunoreceptor-tyrosine-based activation motif intracytoplasmic tail facilitates DENV infection, whereas FcγRIIb inhibits infection [22, 23]. However, the assessment of FcγRIIb was accomplished via transfection of this molecule into cells that naturally expressed FcγRIIIa. Study of how physiological expression levels of both FcγRIIIa and FcγRIIb affect the competition for the binding of available dengue immune complexes and the resultant neutralization and/or enhancement of DENV infection would provide a more relevant model. The additional impact of the presence of DENV coreceptors can also be assessed by using cells such as U937 DC-SIGN that coexpress DC-SIGN and both isoforms of FcγRII.

Another limitation of the study was that assessment of the in vitro-enhancing potential of the Ab response against CYD-TDV-generated Abs was performed on samples coming from immunogenicity studies and not from efficacy ones. Addressing this question beyond the active phase follow up of these efficacy trials would be of interest to determine whether these patterns are maintained and how they track with long-term efficacy and safety. Recent data from the first year of long-term follow up of the 2 phase 3 efficacy studies and from the first 2 years of the phase 2b CYD-TDV trial showed an increased incidence of hospitalized cases among children under the age of 9 [38]. Different hypotheses have been raised to explain these observations [38], and although no link between in vitro enhancement and poor efficacy could be made from the present study with CV1 cells, further in vitro assessment of both the enhancing potential of the Ab response and a detailed study of its qualitative characteristics may be valuable, with particular focus on the age of the subject.

## CONCLUSIONS

In conclusion, sera from CYD-TDV-vaccinated subjects suggest that the lack of correlation between the magnitude of the PRNT<sub>50</sub> GMT values and the level of efficacy measured against DENV1 and DENV2 may not be related to enhancing Abs against these serotypes as determined in a CV-1-FcγRIIa-based neutralization assay. Further analysis of the anti-DENV immune response will likely use a multifaceted approach involving continued development of more physiologically relevant neutralization models to supplement Vero PRNT assays, identification of homotypic and heterotypic (cross-reactive) Abs, and their contribution to the immune response and protection. The combination of several approaches may further illuminate understanding of immune responses with respect to dengue vaccine efficacy.

## Supplementary Material

Supplementary material is available online at Open Forum Infectious Diseases (<http://OpenForumInfectiousDiseases.oxfordjournals.org/>).

## Acknowledgments

We thank Jack Schlesinger for providing the CV-1 cell lines and for helpful discussions. In addition, we thank the volunteers from the clinical trials and Diane van der Vliet, Gustavo Dayan, and Isabelle Darlavoix for helping organize access to these samples. We also thank Jo-Ann West for providing critical reading and editorial support of the manuscript.

**Financial support.** This work was supported by Sanofi Pasteur.

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

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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