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Short Communication

Neutralization of dengue virus in the presence of Fc receptor-mediated phagocytosis distinguishes serotype-specific from cross-neutralizing antibodies

Ryan S.L. Wu [a], Kuan Rong Chan [b], Hwee Cheng Tan [a], Angelia Chow [a], John C. Allen Jr. [c], Eng Eong Ooi [a,*]

[a] Program in Emerging Infectious Diseases, Duke-NUS Graduate Medical School, 169854 Singapore, Singapore
[b] National University of Singapore Graduate School, National University of Singapore, 117456 Singapore, Singapore
[c] Center for Quantitative Medicine, Duke-NUS Graduate Medical School, 169854 Singapore, Singapore

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ABSTRACT

Although several vaccine candidates are presently in various phases of clinical trials, the field still lacks an effective tool to determine protective immunity. The presence of cross-neutralizing antibodies limits a serological approach to identify the etiology and distinguish lifelong from short-lived humoral protection. A recent study indicated that cross-reactive but not serotype-specific antibodies require high antibody concentration to co-ligate FcγRIIB and inhibit infection. Here, we tested if these differences could allow us to distinguish serotype-specific from cross-neutralizing antibodies. Using 30 blinded early convalescent serum samples from patients with virologically confirmed dengue, we demonstrate that neutralization in the presence of FcγR-mediated phagocytosis in THP-1 correctly identifies the DENV serotype of the infection in 93.3% of the cases compared to 76.7% with plaque reduction neutralization test. Our findings could provide a new approach for evaluating DENV neutralization and suggest that in addition to blocking specific ligand–receptor interactions for viral entry, antibodies must prevent viral uncoating during FcγR-mediated phagocytosis for complete humoral protection.

The re-emergence of dengue throughout the tropical world continues unabated without sustainable preventative measures. The presence of four antigenically distinct dengue virus (DENV) serotypes has complicated vaccine development. In particular, the possibility of enhanced infection by non- or sub-neutralizing levels of antibodies necessitates that any vaccine must protect against all four serotypes. Furthermore, there is also a lack of an effective surrogate marker of protective immunity. The plaque reduction neutralization test (PRNT) and various adaptations of this test have been used to measure neutralizing antibody titers and infer immunity (Putnak et al., 2008; Roehrig et al., 2008). However, the presence of cross-neutralizing antibodies especially following a secondary infection with a heterologous DENV serotype or flavivirus vaccination limits the ability of PRNT to serve as a surrogate marker for humoral immunity (Endy et al., 2004). Understanding the requirements for humoral immunity could thus pave the way for vaccine and therapeutic antibody development.

We recently demonstrated a mechanistic role for FcγRIIB in inhibiting phagocytosis of antibody-opsonized DENV (Chan et al., 2011). Using a limited number of convalescent serum samples obtained within 3 weeks from illness onset as well as humanized monoclonal antibody, we observed that neutralization of homologous DENV serotypes occurred at serum dilutions that permitted FcγR-mediated phagocytosis but neutralization of heterologous DENV serotypes only occurred at serum dilutions that aggregates DENV to co-ligate FcγRIIB, resulting in downstream signaling that inhibits phagocytosis (Chan et al., 2011). This finding suggests that serotype-specific neutralization can be differentiated from cross-reactive antibodies by assessing for neutralization in the presence of FcγR-mediated phagocytosis. This is important as long-lasting humoral immunity following DENV infection is directed at the homologous but not heterologous serotypes (Sabin, 1952). Here, we report a clinical validation of detecting DENV neutralization in the presence of FcγR-mediated phagocytosis.

We took advantage of the known presence of cross-neutralizing antibodies in early convalescence following a primary DENV infection (Beltramello et al., 2010; Dejnirattisai et al., 2010), which would enable us to compare a serological determination of the serotype of infection with the virological findings in the acute sera and determine its accuracy, unequivocally, for this study. We designed an investigator-blinded test of early convalescent serum samples obtained from patients with virologically confirmed DENV infection. A schematic illustration of the study approach is shown in Fig. 1. Human sera used in this study were obtained from the early dengue infection and control (EDEN) study as previously described (Low et al., 2006) and approved by the National Healthcare Group Domain Specific Review Board (DSRB B/05/013). These

* Corresponding author. Address: Duke-NUS Graduate Medical School, Program in Emerging Infectious Diseases, 8 College Road, 169857 Singapore, Singapore. Tel.: +65 6516 8594; fax: +65 6221 2529.
E-mail address: engeong.ooi@duke-nus.edu.sg (E.E. Ooi).

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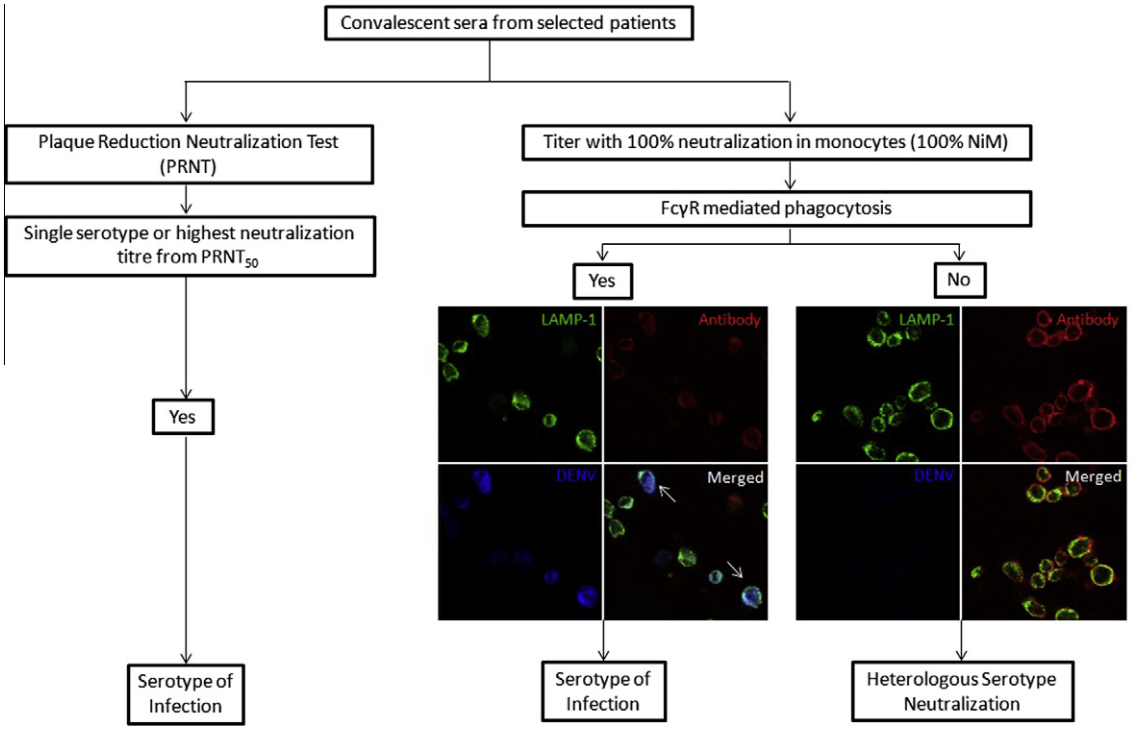

Fig. 1. Workflow of the study. $PRNT_{50}$ and 100% DENV neutralization in THP-1 were determined for all 30 early convalescent samples. The highest neutralization titer from $PRNT_{50}$/100% neutralization in THP-1 was taken to indicate the serotype of infection as conventionally used by other studies. Furthermore, based on 100% neutralization in THP-1, confocal immunofluorescence was performed to determine the presence or absence of co-localization of labeled antibody-virus complexes. Presence of co-localization of antibody-virus complexes with LAMP-1 was taken to indicate the presence of serotype-specific antibodies (white arrows), while inhibition of FcγR-mediated phagocytosis was taken to signify the presence of only cross-reactive antibodies. Green – LAMP-1, red – human IgG, blue – DiD labeled DENV.

samples were from adult patients (age > 21 years) who provided written informed consent for the use of material and clinical information for research purposes. Patients included in this study had positive RT-PCR findings but negative anti-dengue IgG in the acute serum samples (obtained within 72 h from illness onset) as measured by ELISA (PanBio). The presence of pre-existing anti-flavivirus antibodies such as those against Japanese encephalitis virus, yellow fever and West Nile virus was not assessed although the ELISA would have detected cross-reactive antibodies from prior infection or vaccination with these viruses. *A priori* statistical calculation using Wilson's approach for calculating two sided confidence intervals, indicated that a sample size of 30 would provide a proportion estimate of 0.9 with a pre-set 90% confidence interval width of less than 0.20 (0.77,0.96) (PASS © 2010 Software). Hence, by convenience sampling, 30 convalescent sera were selected and coded by one of the co-authors (AC). Subsequent studies were carried out by all other authors blinded to the findings in the acute sera. The selected sera were collected at a median of 18 days (minimum 12 days, maximum 32 days) from illness onset.

$PRNT_{50}$ and DENV neutralization in THP-1 were carried out on the convalescent sera as described previously (Chan et al., 2011). In these experiments, DENV-1 (07K2402DK1), DENV-2 (ST), DENV-3 (05K802DK1) and DENV-4 (05K2270DK1) were used. To determine $PRNT_{50}$ titers, serial 2-fold dilutions of the sera were incubated with 40 pfu of DENV at 37 °C for 1 h before adding to BHK-21. The serotype with the highest dilution that neutralized 50% of the plaque forming units was interpreted as causative of the acute infection. Complete (100%) DENV neutralization in THP-1 was determined by incubating serial 2-fold dilutions of sera with DENV, before adding to THP-1 at a multiplicity of infection of 10. After 72 h incubation, plaque assay on BHK-21 was performed on the THP-1 culture supernatant. The serotype with the highest dilution that neutralized 100% of DENV was interpreted as causative of the acute infection. We also reacted sera with DiD (1,1-dioctadecyl-3,3,3,3-tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate salt)-labeled DENV (van der Schaar et al., 2007), at dilutions where 100% neutralization of DENV was seen in THP-1 and performed confocal immunofluorescence microscopy to assess for FcγR-mediated phagocytosis at 30 min post-inoculation (Fig. 1). Complete DENV neutralization with FcγR-mediated phagocytosis was taken as the serotype of the acute infection (Fig. 1). The RT-PCR findings in the respective acute sera were un-blinded only upon completion of the serological analyses.

Of the 30 convalescent samples, only eight (26.7%) showed $PRNT_{50}$ to a single serotype. Similarly, these eight sera displayed neutralizing titers to a single serotype in THP-1, all of which neutralized DENV in the presence of FcγR-mediated phagocytosis (Table 1). Among the remaining 22 convalescent sera, the highest $PRNT_{50}$ titer was consistent with the serotype detected by RT-PCR in the acute sera in 15 cases (68.2%, 95% confidence interval (95% CI) 45.0–86.1%). In the 11 samples where the highest $PRNT_{50}$ titer was at least 4-fold or higher than those of the other serotypes, the highest $PRNT_{50}$ titer was consistent with the serotype of the infection. However, in the other 11 of the samples that showed (i) identical titers to two serotypes or (ii) only 2-fold difference between the highest and the next highest titer, only 4 (36%) were consistent with the serotype of the infection (Table 1).

Using the highest dilution that mediated 100% DENV neutralization in THP-1, only 13 out of the 22 cases correctly identified the serotype of infection (59.1%, 95% CI 36.4–79.3%) (Table 1). Confocal imaging, however, clarified the serotype of the acute infection, where 20 out of the 22 cases (90.9%, 95% CI 70.8–98.9%) showed complete DENV neutralization in the presence of FcγR-mediated phagocytosis (Table 1). Overall, the accuracy of $PRNT_{50}$, 100% neutralization in THP-1 and confocal imaging were 76.7% (95% CI 57.7–90.1%), 70.0% (95% CI 50.6–85.3%) and 93.3% (95% CI

Table 1
Correlation of $PRNT_{50}$, 100% dengue neutralization in THP-1 and FcγR-mediated phagocytosis with serotype of infection as determined by RT-PCR for DENV in the corresponding acute serum samples.

	No. of early convalescent sera	No. of sera correlated with serotype of infection		
		$PRNT_{50}$	100% neutralization in THP-1	FcγR-mediated phagocytosis
Homotypic	8	8	8	8
Heterotypic	22	15	13	20
Total	30	23	21	28

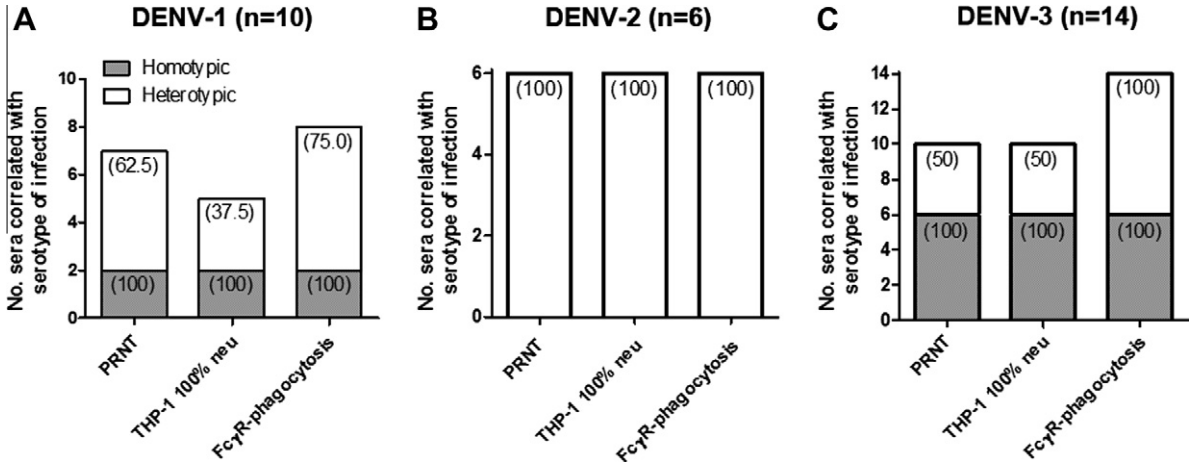

Fig. 2. Accuracy of $PRNT_{50}$ and 100% neutralization in THP-1 with or without observing for Fc-receptor-mediated phagocytosis in identifying the serotype of the acute infection. Number of patient samples predicted correctly for (A) DENV-1 (n = 10), (B) DENV-2 (n = 6) and (C) DENV-3 (n = 14) using either $PRNT_{50}$, 100% neutralization in THP-1 or observing for FcγR-mediated phagocytosis. Patient samples with only one neutralization titer are classified as homotypic (Grey bars) and samples with more than one neutralization titers are classified as heterotypic (White bars). In brackets are percentages in which the serotype determined serologically correlated with the DENV serotype detected by RT-PCR in the respective acute serum samples.

77.9–99.2%), respectively. Fig. 2 summarizes the results from the three different methods used in our study by DENV serotype. None of our patients were infected with DENV-4.

PRNT and most other neutralization assays have used epithelial cells, such as Vero or BHK-21 as host cells for DENV infection. These cells neither express FcγR nor are they the primary targets of DENV *in vivo*. Monocytes, on the other hand, play a central role in dengue virus replication (Durbin et al., 2008; Halstead, 1988) as well as the clearance of immune complexes. Using THP-1, which was derived from a patient with acute monocytic leukemia, we had observed that convalescent serum could only neutralize the homologous serotypes in the presence of FcγR-mediated phagocytosis (Chan et al., 2011). Our present finding supports this hypothesis and demonstrates that such an approach could be used to determine the serotype of the infection. This approach could be useful in assessing the efficacy of vaccination to each of the four DENV serotypes. As the tetravalent formulation of candidate dengue vaccines would elicit pan-dengue antibodies, clarifying whether these antibodies are able to neutralize each of the four DENV serotypes in the presence of FcγR phagocytosis, similar to antibodies generated following an acute infection, could inform on whether vaccination is likely to result in long-term serotype-specific immunity.

Our current findings also raise important questions. It is not evident why neutralization of heterologous serotypes could not occur in the presence of FcγR-mediated phagocytosis. It is possible that cross-reactive antibodies need higher amounts of antibodies to fulfill the stoichiometric requirement for DENV neutralization compared to serotype-specific antibodies (Pierson et al., 2007) and these antibody concentrations coincide with that which aggregates DENV for FcγRIIB co-ligation (Chan et al., 2011). It is also possible that the cross-reactive antibodies to DENV antigens have lower binding affinities that are compromised in the low pH environment within phagosomes. Indeed, serotype-specific antibodies appear to be more potent in DENV neutralization although cross-reactive antibodies were more abundant in convalescent sera (de Alwis et al., 2012). Hence, we suggest that in addition to blocking specific ligand-receptor interactions for viral entry, antibodies must prevent viral uncoating during FcγR-mediated phagocytosis for complete humoral protection. Clarifying this could be important for identifying suitable antibodies for therapeutic development (de Alwis et al., 2011, 2012; Teoh et al., 2012).

In conclusion, determining if virus neutralization occurs in the presence of FcγR-mediated phagocytosis can clarify the serotype of the DENV infection serologically.

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