MAJOR ARTICLE

Preexisting Neutralizing Antibody Responses Distinguish Clinically Inapparent and Apparent Dengue Virus Infections in a Sri Lankan Pediatric Cohort

Kizzmekia S. Corbett,[1] Leah Katzelnick,[1,2] Hasitha Tissera,[3] Ananda Amerasinghe,[3] Aruna Dharshan de Silva,[4] and Aravinda M. de Silva[1]

[1]Department of Microbiology and Immunology, University of North Carolina School of Medicine, Chapel Hill; [2]Centre of Pathogen Evolution, University of Cambridge, United Kingdom; [3]Epidemiology Unit, Ministry of Health, and [4]Genetech Research Institute, Colombo, Sri Lanka

Dengue viruses (DENVs) are mosquito-borne flaviviruses that infect humans. The clinical presentation of DENV infection ranges from inapparent infection to dengue hemorrhagic fever and dengue shock syndrome. We analyzed samples from a pediatric dengue cohort study in Sri Lanka to explore whether antibody responses differentiated clinically apparent infections from clinically inapparent infections. In DENV-naive individuals exposed to primary DENV infections, we observed no difference in the quantity or quality of acquired antibodies between inapparent and apparent infections. Children who experienced primary infections had broad, serotype–cross-neutralizing antibody responses that narrowed in breadth to a single serotype over a 12-month period after infection. In DENV immune children who were experiencing a repeat infection, we observed a strong association between preexisting neutralizing antibodies and clinical outcome. Notably, children with preexisting monospecific neutralizing antibody responses were more likely to develop fever than children with cross-neutralizing responses. Preexisting DENV neutralizing antibodies are correlated with protection from dengue disease.

Keywords. dengue virus; neutralizing antibody; inapparent dengue; dengue fever; Sri Lanka.

Dengue virus (DENV) is a positive-stranded RNA virus that is transmitted to humans via the bite of *Aedes* mosquitoes. DENVs exist as 4 serotypes, DENV1–4, which circulate in tropical and subtropical regions. Currently, over two thirds of the world's population is at risk of being exposed to DENV [1, 2]. A recent study estimates that 390 million DENV infections occur globally each year, rendering DENV the most common mosquito-borne viral pathogen among humans [3].

Natural human DENV infection can result in clinically inapparent or apparent infections. Apparent infections, which account for less than half of total DENV infections, manifest as mild dengue fever, severe dengue hemorrhagic fever, or potentially fatal dengue shock syndrome [3]. The most significant risk factor for severe disease is previous DENV infection: an individual experiencing secondary infection with a heterologous DENV serotype faces greater risk of developing severe disease than someone experiencing primary infection [4–8]. Antibody-dependent enhancement is the leading explanation for the increased risk of severe dengue disease following reinfection. The antibody-dependent enhancement theory postulates that primary DENV infection induces cross-reactive nonneutralizing antibodies that promote entry of DENV particles into FcγR-bearing cells upon secondary infection with a heterologous DENV serotype. This phenomenon is believed to result in increased cellular viral burden and subsequent severe disease [9–11].

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Correspondence: Aravinda M. de Silva, PhD, Department of Microbiology and Immunology, University of North Carolina School of Medicine, 160 Dental Circle, Chapel Hill, NC 27599 (desilva@med.unc.edu).

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Many studies have been performed to examine the role of antibodies in severe dengue disease [10, 12–16]. A topic that has been less studied is a comparison of the role of antibodies in clinically inapparent versus clinically apparent DENV infection [17–19]. In this study, we used sera collected from a prospective pediatric fever surveillance study in Colombo, Sri Lanka [20], to test our hypothesis that antibody responses are linked to the development of inapparent and apparent DENV infections.

MATERIALS AND METHODS

Human Subjects Protocol Approval

Ethical approval for this research was obtained from the Ethical Review Committee of the Faculty of Medicine, University of Colombo, and the Institutional Research Board of the International Vaccine Institute, Seoul, Korea. The University of North Carolina (UNC) institutional review board determined that its approval was not required because participating UNC investigators were not involved in human subjects research. Only children whose parents or legal guardians provided written informed consent were enrolled in the study.

Cell Lines and Viruses

U937 monocytic cells stably transfected with the gene encoding DC-SIGN (U937–DC-SIGN cells) were maintained in Roswell Park Memorial Institute medium supplemented with 5% fetal bovine serum, 1% L-glutamine, 1% penicillin/streptomycin, 1% nonessential amino acids, and 0.05 mM β-mercaptoethanol. The C6/36-derived World Health Organization reference DENV strains DENV1 (West Pac 74), DENV2 (S-16803), DENV3 (CH 53598), and DENV4 (TVP-376) were used in all infection-based experiments.

Sample Collection

Surveillance and sample collection methods were previously detailed [20, 21]. Briefly, between November 2008 and January 2010, blood samples were collected from 799 children aged ≤12 years in Colombo, at enrollment (baseline) and 12 months later (follow-up). In addition, among children who experienced febrile illness, blood samples were obtained upon fever onset (acute phase specimens) and ≥10 days following fever dissipation (convalescent phase specimens) [20]. Blood samples were stored as dried blood spots (DBS) on protein saver cards (Whatman, United Kingdom; ID Biological Systems, Greenville, SC) [22, 23] or were centrifuged and stored as plasma.

Elution of Antibodies From DBS

DBS diluent volume was determined on the basis of standard plasma dilutions in pilot experiments, using matched DBS and plasma obtained from our dengue traveler cohort [24]. Antibodies were eluted from DBS by submerging filter paper in diluent appropriate for subsequent assay. DBS/diluent mixtures were incubated at 37°C for 2 hours. Resulting DBS eluates (sera) were used in immunoglobulin G (IgG), immunoglobulin M (IgM), and neutralization assays, as described below.

Detection of DENV-Specific IgG and IgM Antibodies

Immunoassays for detection of DENV-specific IgG and IgM antibodies were performed as previously described [25, 26]. Sera dilutions of 1:100 and 1:50 were evaluated in IgG and IgM enzyme-linked immunosorbent assays (ELISAs), respectively. Cutoffs for IgM and IgG positivity were determined on the basis of positive control samples and, where applicable, are represented as standard deviations relative to normal human sera (NHS) controls (n = 10; pooled).

Detection of DENV Neutralizing Antibodies

Sera were assessed for the presence of neutralizing antibodies against each DENV serotype, using a flow cytometry–based neutralization assay with U937 DC-SIGN cells, as previously described [27, 28]. Sera dilutions were 1:40–10 240 in 4-fold increments, unless otherwise noted. Samples were run on GUAVA easyCyte HT and analyzed on GUAVASoft software (Millipore, Billerica, MA). Sigmoidal curves were generated using Prism v4.0 (GraphPad Software, La Jolla, CA). The 50% neutralization titer (NT_{50}) was calculated as the serum dilution that neutralizes 50% of each DENV serotype. The seroconversion threshold was set to ≥50% neutralization at a ≥1:40 serum dilution.

Laboratory Confirmation of DENV Infections

To identify clinically apparent DENV infections, acute- and convalescent-phase samples were collected from children with fever and tested by polymerase chain reaction and paired IgG and IgM serologic analysis, as previously described [20]. Details of how inapparent DENV infections were detected are specified in the Supplementary Materials. Briefly, all children were screened for increases in neutralizing antibody levels over the study year by testing paired baseline and 12-month follow-up samples (at dilutions of 1:40 and 1:640) by means of a neutralization assay (Supplementary Table 1). Cases were then confirmed by a neutralization assay with the full serum dilution series (Supplementary Figure 1). DENV infections occurring in children who did not have laboratory-confirmed apparent dengue fever during the study year were classified as inapparent DENV infections. Dengue cases were further classified as primary or secondary on the basis of the affected individual's baseline DENV naive or immune status, respectively. Notably, all DENV cases in this study were confirmed by a gold-standard neutralization assay [27, 28]. Supplementary Table 2 displays the sensitivity and specificity of the IgG ELISA to detect DENV cases, calculated using results of the gold-standard neutralization assay. Although it is possible that some inapparent infections were missed dengue fever cases, efforts were made to ascertain case detection by educating parents and having a

Table 1. Summary of Confirmed Cases of Dengue Virus (DENV) Infection in a Sri Lankan Pediatric Cohort [20]

	DENV Infection, Children, No.				
	Primary Infection (n = 35)		Secondary Infection (n = 32)		
Baseline Status[a]	Inapparent[b]	Apparent[c]	Inapparent[d]	Apparent[e]	No Infection, Children, No. (n = 732)[f]
DENV naive (n = 358)	20	15	NA	NA	323
DENV immune (n = 441)	NA	NA	20	12	409

Abbreviations: IgG, immunoglobulin G; NA, not applicable; RT-PCR, reverse transcription polymerase chain reaction.

[a] Determined by testing a baseline sample for DENV IgG, using an enzyme-linked immunosorbent assay.

[b] Determined by detection of DENV IgG seroconversion at follow-up and confirmed by neutralization curves.

[c] Confirmed by RT-PCR and serologic analyses indicating the presence of DENV during fever and further classified as primary on the basis of baseline DENV IgG–naive status.

[d] Determined by a 2-dilution neutralization screen and confirmed by neutralization curves.

[e] Confirmed by RT-PCR and serologic analyses indicating the presence of DENV during fever and further classified secondary based on baseline DENV IgG immune status.

[f] Defined as children who did not acquire DENV infection during the study period.

study team visit each house weekly [21]. Loss to follow-up was minimal: of 800 children enrolled, only 1 child withdrew during the 12 months [20, 21].

Quantitative Analysis of the Breadth of Neutralizing Antibody Responses

The ability of a serum specimen to neutralize each DENV serotype was calculated as $\log_2$[reciprocal NT_{50} / 10]. The neutralization breadth of a serum specimen was expressed as the average difference in titer between the virus best neutralized by that specimen (the maximum titer) and the titers for each of the other 3 DENV types. Thus, a value close to 0 indicates that the serum specimen neutralized all DENV types similarly, while a higher value indicates that the specimen neutralized one DENV type better than it neutralized the other 3 types.

Statistical Analyses

To compare DENV IgG levels between inapparent and apparent groups, we used unpaired Student *t* tests. Repeated measure 1-way analysis of variance (ANOVA) with the Tukey multiple comparison post hoc test was used to compare DENV IgG levels in paired baseline, acute phase, convalescent phase, and follow-up groups. To compare the breadth of the neutralizing antibody responses between groups, we performed Fisher exact tests, defining outcome categories as "monotypic" (ie, seroconversion to 1 serotype) and "heterotypic" (ie, seroconversion to ≥2 serotypes). The Pearson product-moment correlation test was used to determine the linear relationship between elapsed time and neutralization breadth. In the quantitative analysis of neutralization breadth, the Wilcoxon rank sum test was used for statistical comparison between groups, as samples were not normally distributed. To analyze the difference in the mean number of serotypes neutralized at baseline and follow-up for secondary inapparent cases, we used a paired Student *t* test. A 1-way ANOVA with a Tukey multiple comparison post hoc test was used to compare the mean number of serotypes neutralized at baseline, the acute phase, the convalescent phase, and follow-up for secondary apparent cases. For some assays, sample variability exists because of the limited quantity of sample available for the multiple and repeat analysis of the various assays, rather than because of loss at follow-up. *P* values were determined by 2-tailed analyses, unless otherwise specified. Generation of graphs and statistical analyses was completed using Prism v4.0.

RESULTS

We used blood samples from a prospective pediatric dengue cohort study in Colombo to compare antibody responses between children with clinically inapparent dengue infection and those with clinically apparent dengue infection. The cohort population, study design, surveillance methods, and epidemiological findings are detailed elsewhere [20, 21]. As previously reported, we identified 35 primary infections and 32 secondary and repeat infections over the 12-month study period (Table 1) [20]. Forty of the infections (20 primary infections and 20 secondary and repeat infections) were classified as inapparent because they occurred in children who did not have laboratory-confirmed dengue fever during the 12-month period of active disease surveillance.

Antibody Responses Following Primary DENV Infections

First, we characterized antibody responses following primary DENV exposure. With apparent infections, for which we knew the date of fever onset, we found a significant negative correlation between the time that elapsed after infection and the breadth of neutralization across the 4 serotypes (Pearson $r = -0.69$; $P < .01$): children who experienced dengue fever recently had neutralizing antibody responses against a greater

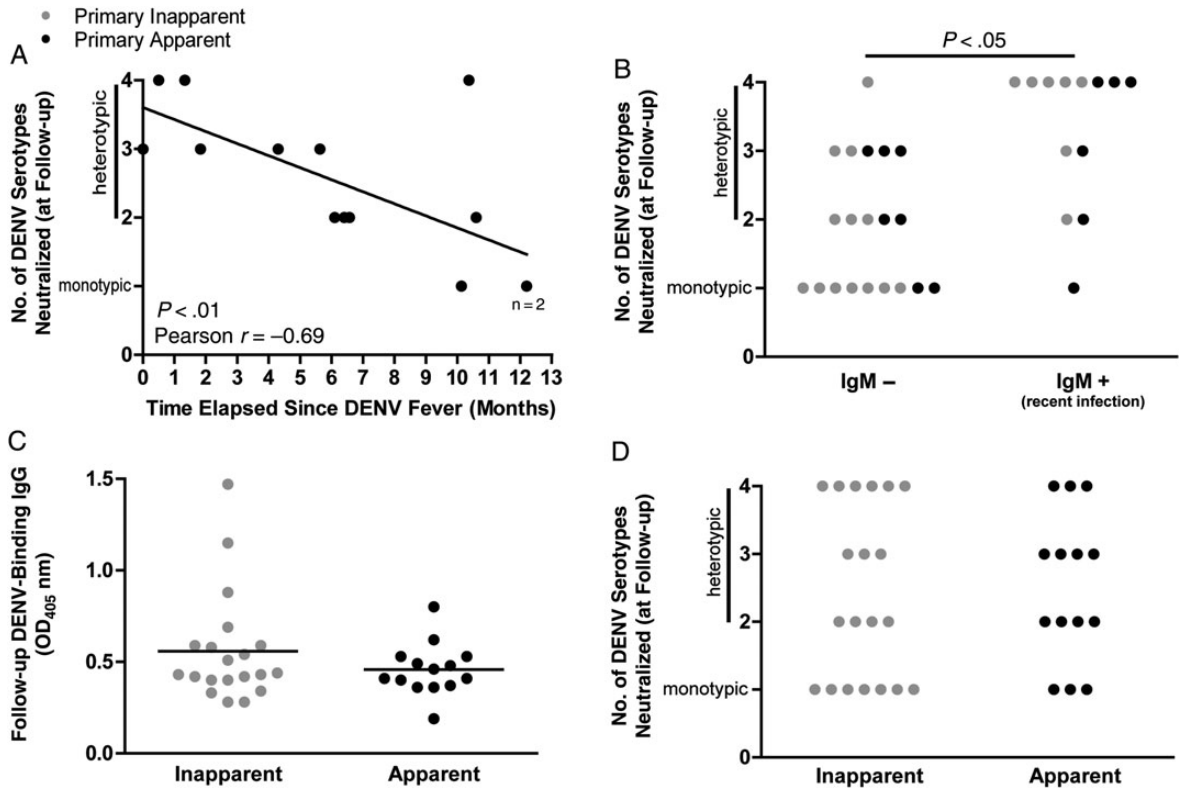

Figure 1. Antibody responses following primary dengue virus infections. *A*, *B*, and *D*, Follow-up sera from children with primary dengue virus (DENV) infections were subjected to neutralization assays at dilutions of 1:40–10 240 in 4-fold increments against DENV1–4. Sigmoidal curves were generated, and 50% neutralization titers were calculated for each serotype. The neutralization seroconversion threshold was set to ≥50% neutralization at ≥1:40 serum dilution for each DENV serotype. *A*, For primary apparent infections, the time that had elapsed since DENV fever onset was determined as amount of time between date of acute fever onset and date of follow-up sample collection. The line was generated by linear regression analysis. Correlation between neutralization breath and elapsed time was calculated by the Pearson product-moment correlation test. *B*, Sera were subjected to an immunoglobulin M (IgM) enzyme-linked immunosorbent assay (ELISA) at a 1:50 dilution; IgM positivity was determined to be 5 SDs greater than the value for normal human sera (n = 10; pooled). Groups were compared using the Fisher exact test, with outcome categories defined as "monotypic" (ie, seroconversion to 1 serotype) and "heterotypic" (ie, seroconversion to ≥2 serotypes). *C*, Sera were subjected to an immunoglobulin G ELISA at a 1:100 dilution. Lines represent mean OD_{405nm} values. Means were found to be insignificant by the unpaired Student *t* test. *D*, Number of DENV serotypes neutralized in children with inapparent or apparent infection. Groups were compared using the Fisher exact test, with outcome categories defined as monotypic and heterotypic as described above. For all graphs, each dot represents mean value for an individual serum specimen tested in duplicate, unless otherwise noted.

number of serotypes than children who experienced dengue fever early in the study year (Figure 1*A*). For all primary infections, both inapparent and apparent, we tested follow-up sera for presence of DENV IgM, which is indicative of recent infection (ie, infection within the past 6 months) [29]. Serotype–cross-neutralizing sera were more likely than monotypic sera to be IgM positive ($P < .05$; Figure 1*B*), further confirming the association between recent primary exposure and broad neutralization.

We then compared acquired DENV-specific antibody responses in children with primary inapparent infection and those with primary apparent infection. We found no difference in total levels of DENV-binding IgG (Figure 1*C*) or the ability of acquired antibodies to neutralize the 4 DENV serotypes (Figure 1*D*). Both primary inapparent and apparent DENV infections induced neutralizing antibody responses that were monotypic in some cases and heterotypic in other cases. Together, these data indicate that children experiencing primary inapparent infection and those experiencing apparent infections develop similar DENV-specific antibody responses. Furthermore, primary DENV infection stimulates a DENV serotype–cross-neutralizing response that evolves over time into a more monotypic response.

Temporal Analysis of Antibody Responses Following Repeat DENV Infections

After seeing temporal changes in antibody responses following primary infections, we measured changes in DENV-specific antibodies as a function of time following repeat DENV infections. We focused on apparent infections for which dates of actual infection were known (Figure 2*A*). There was a significant

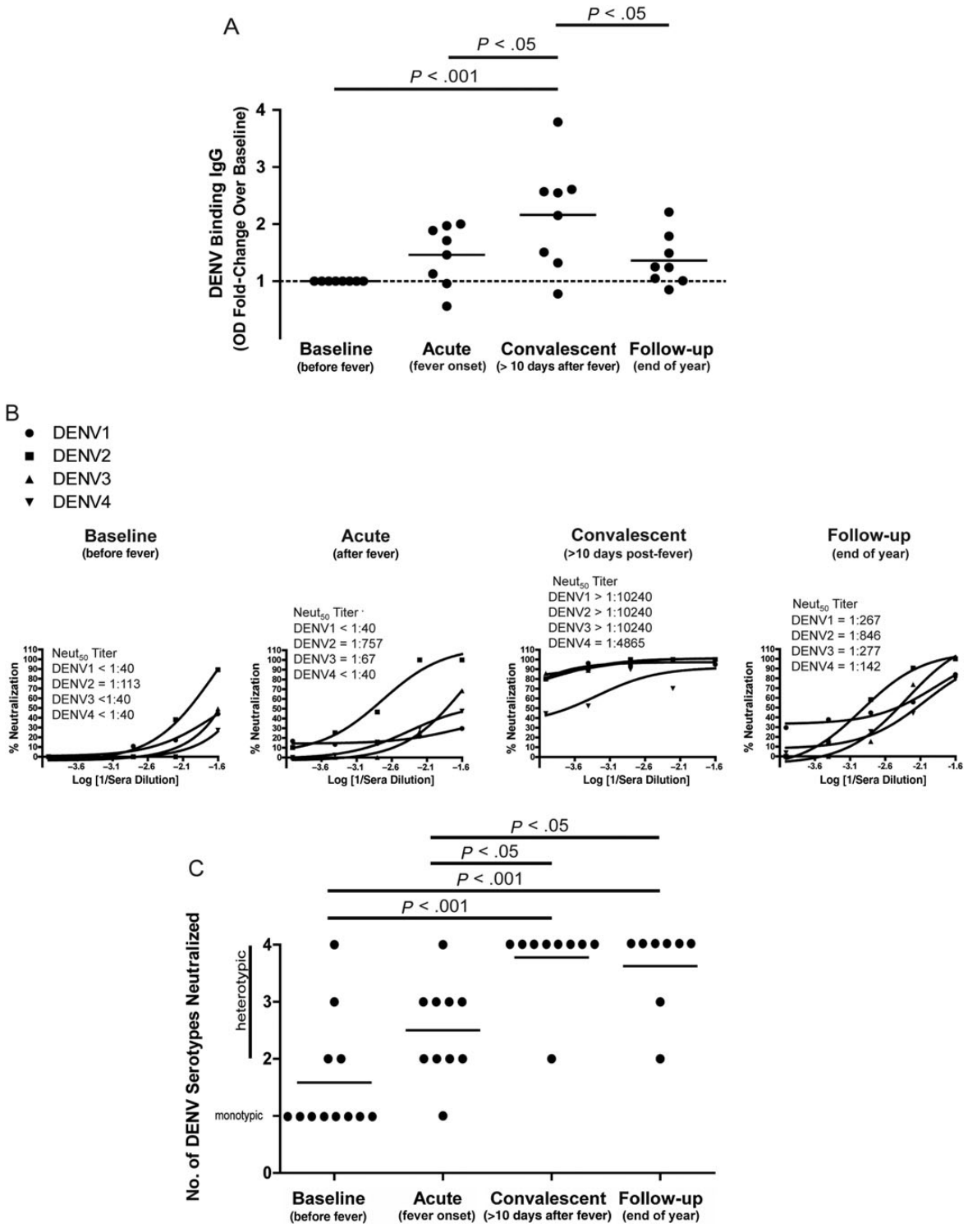

Figure 2. Temporal analysis of antibody responses following repeat dengue virus (DENV) infections. Paired baseline, acute phase, convalescent phase, and follow-up sera (when available) from repeat apparent DENV infections were subjected to an immunoglobulin G (IgG) enzyme-linked immunosorbent assay at a 1:100 dilution (*A*) and neutralization assays at dilutions of 1:40–10 240 in 4-fold increments against DENV1–4 (*B* and *C*). *A*, DENV IgG OD values were calculated as fold change with respect to baseline OD values. Lines depict mean ODs, which were all compared by repeated measure 1-way analysis of variance (ANOVA) with the Tukey multiple comparison post hoc test. *B*, Sigmoidal curves were generated, and 50% neutralization titers (NT_{50}) were calculated for each serotype. The graphs depict temporal sera collected from 1 child. *C*, The neutralization/seroconversion threshold was set to ≥50% neutralization at a serum dilution of ≥1:40 for each DENV serotype. Lines show the mean number of DENV serotypes neutralized at each time point. Time points were compared by 1-way ANOVA with the Tukey multiple comparison post hoc test. For all graphs, each dot represents the mean value for an individual serum specimen tested in duplicate.

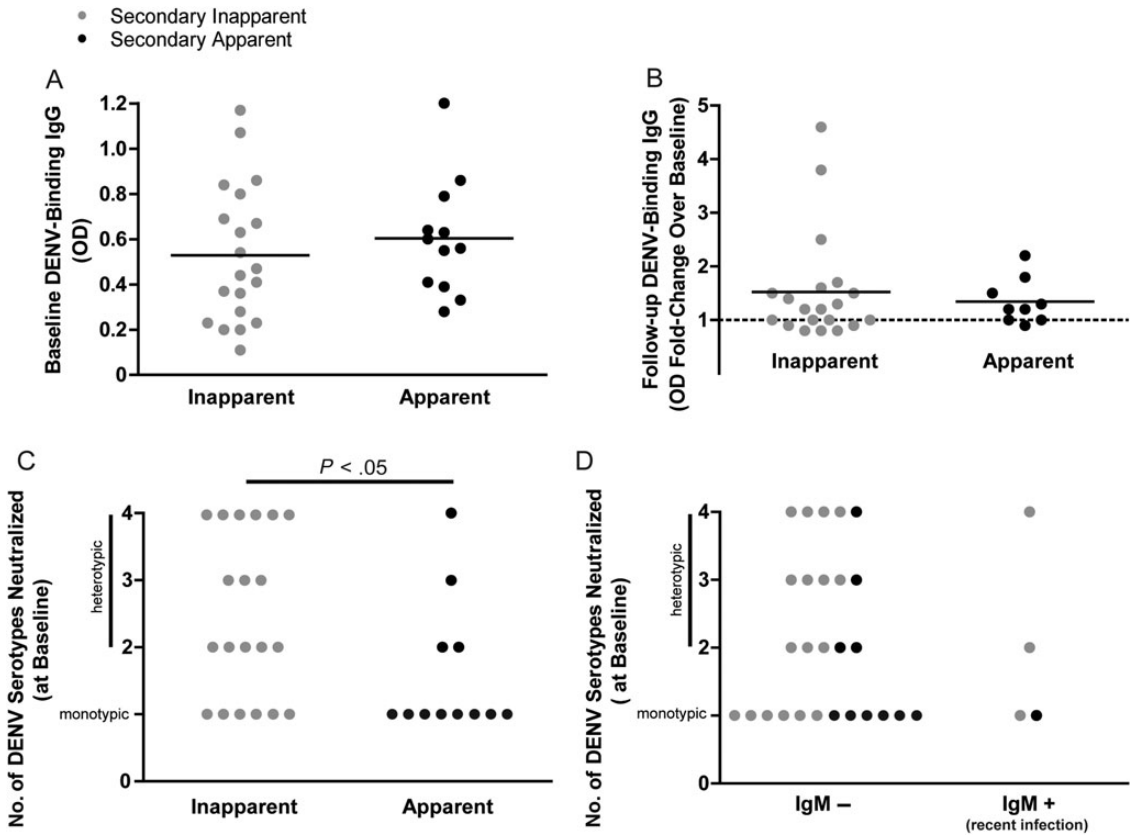

Figure 3. Antibody responses of repeat inapparent and apparent dengue infections. Sera (when available) from repeat dengue virus (DENV) infections were subjected to an immunoglobulin G (IgG) enzyme-linked immunosorbent assay (ELISA; *A* and *B*), neutralization tests (*C* and *D*), and an immunoglobulin M (IgM) capture ELISA (*D*). *A* and *B*, Paired baseline and follow-up sera were used at a 1:100 dilution. *A*, For baseline sera, mean OD_{405nm} values were calculated (represented by lines). *B*, For follow-up sera, ODs are represented as fold-change over baseline ODs. *A* and *B*, Differences in mean ODs between inapparent and apparent cases were found to be insignificant by the unpaired Student *t* test. *C* and *D*, Baseline sera were subjected to neutralization assays at dilutions of 1:40–10 240 in 4-fold increments against DENV1–4. Sigmoidal curves were generated, and 50% neutralization titers were calculated for each serotype. The neutralization/seroconversion threshold was set to ≥50% neutralization at a serum dilution of ≥1:40 for each DENV serotype. *B*, Baseline sera were also subjected to IgM ELISA at a 1:50 dilution; IgM positivity was determined to be 5 SDs greater than the value for normal human sera (n = 10; pooled). *C* and *D*, Groups were compared using the Fisher exact test, with outcome categories defined as "monotypic" (ie, seroconversion to one serotype) and "heterotypic" (ie, seroconversion to ≥2 serotypes).

increase in DENV IgG levels during the early convalescent phase (approximately 2–4 weeks after infection), compared with levels at baseline ($P < .001$) and during the acute phase ($P < .05$). However, these elevated IgG levels decreased to preinfection and acute-phase levels by the end of the study year (follow-up). Neutralization curves for sera obtained at different times from individuals with apparent reinfections revealed boosting of neutralizing antibody titer against the serotype of previous infection (Figure 2*B*). At the convalescent phase, there was a robust increase in neutralizing antibody titer against multiple DENV serotypes. Although the overall magnitude of the neutralizing antibody response was reduced at follow-up, the response remained broadly cross-neutralizing (Figure 2*B* and 2*C*). Our complete analysis of neutralization breadth in apparent repeat infections revealed a significant and stable increase that was maintained through the end of the study (ie, throughout the follow-up period), compared with levels before infection (ie, at baseline; $P < .001$) and during the acute phase ($P < .05$; Figure 2*C*). Thus, for repeat infections, although the overall quantity of DENV-specific IgG antibody increased and then decreased back to levels before infection and during the acute phase, the breath of neutralization was increased and maintained over the duration of the study year.

Comparison of Antibody Responses of Repeat Inapparent Versus Apparent DENV Infections

We then compared antibody responses in children experiencing repeat inapparent infections to those in children with repeat apparent infections. We found no difference in preexisting (ie, levels measured at baseline; Figure 3*A*) or acquired (ie, levels measured during follow-up; Figure 3*B*) total DENV-specific IgG levels between these 2 groups. However, when we compared

Table 2. Quantitative Analysis of the Breadth of the Dengue Virus (DENV) Neutralizing Antibody Responses

Variable	Primary Infection, Median (IQR)	Repeat Infection, Median (IQR)		*P* Value
		Inapparent	Apparent	
Baseline	NA	3.21 (2.20–4.01)	5.10 (3.53–5.66)	.03[a]
Follow-up	3.91 (2.52–4.51)[b]	3.17 (1.74–4.91)	1.49 (0.90–2.82)	.04[a]
Maximum titer[c]				
Baseline	NA	4.39 (3.55–5.40)	4.58 (3.78–5.10)	.57[b]
Follow-up	4.39 (3.73–6.69)	6.48 (5.61–7.26)	5.87 (5.03–6.67)	.02[d]

The breadth of response, expressed as $\log_2$[reciprocal NT_{50} / 10], is a measure of the average difference in titer between the DENV serotype best neutralized by a serum specimen (the maximum titer) and the titers for each of the other 3 DENV serotypes. A value close to 0 indicates that the serum specimen neutralized all DENV types similarly, whereas higher values indicate that the serum specimen neutralized one DENV type significantly better than it neutralized the other 3 types.

Abbreviations: IQR, interquartile range; NA, not applicable; NT_{50}, 50% neutralization titer.

[a] By the Wilcoxon rank sum test, for difference between repeat inapparent and apparent infections.

[b] By the Wilcoxon rank sum test, for difference in breadth between primary infections and repeat infections. The difference between primary infections and repeat inapparent infections at baseline was not significant ($P = .3$).

[c] Maximum titer for each individual, regardless of the infecting type, is shown.

[d] By the Kruskal–Wallis test, for difference in maximum titer between primary infections, inapparent repeat infections, and apparent repeat infections.

preexisting neutralizing antibody levels, we found that children who experienced repeat inapparent DENV infections were more likely to have heterotypic neutralizing responses at baseline, compared with children who experienced repeat apparent infections ($P < .05$; Figure 3*C*). To examine whether children with repeat inapparent infections experienced very recent past DENV infections, we tested preinfection sera for the presence of DENV-specific IgM. We did not observe a correlation between the presence of IgM and neutralization breadth or clinical outcome (Figure 3*D*). In fact, 17 of 20 baseline sera (85%) from children who had inapparent repeat infections were DENV IgM negative, indicating that their prior DENV exposures had not occurred in the past 6 months. These data indicate that the neutralization breadth of preinfection antibodies has significant bearing on the clinical outcome of subsequent DENV infection. Furthermore, the difference in neutralization capacity between the group with repeat inapparent infections and the group with repeat apparent infections cannot simply be explained by very recent exposure, based on the presence of IgM.

Quantitative Analysis of Differences in Neutralizing Antibody Responses Between Types

We conducted a direct analysis of the difference in neutralization of DENV types to delve further into the neutralizing antibody responses in the cohort (Table 2). The breadth of response was measured as the difference between the titer of the dominantly neutralized DENV type, assumed to be of the previous infecting type, and the titer of the other 3 types. Similar to our aforementioned findings, we determined that children experiencing repeat apparent DENV infections had more monotypic baseline neutralizing antibody responses (ie, dominant responses to a single DENV serotype) than those who acquired repeat inapparent infections ($P < .05$). Interestingly, a significant difference in postinfection breadth was also observed between inapparent and apparent repeat infections: apparent infections were observed to develop more-balanced responses following infection, compared with inapparent infections ($P < .05$). Thus, children whose repeat infections were inapparent had relatively conserved patterns of neutralization at baseline and follow-up, while children whose repeat infections were apparent transitioned from mostly type-specific neutralization before infection to broad neutralization after their repeat DENV exposure. The degree of the neutralization breadth in this sample was independent of the magnitude of the best-neutralized DENV type before infection. Further, children with inapparent repeat infections and those with apparent repeat infections had similar maximum titers at baseline and follow-up. Notably, after the study year, children with inapparent repeat infections and those with apparent repeat infections had higher titers than children with primary infections ($P < .05$).

DISCUSSION

In this study, we used blood samples collected from a Sri Lankan pediatric dengue cohort to investigate antibody responses in children with clinically inapparent DENV infections and those with clinically apparent DENV infections. A strength of our study is that infections were detected by using a neutralization test to evaluate paired baseline and follow-up blood samples. This approach is not feasible with most cohort studies because of the difficulty of using the DENV neutralization test to analyze large sample panels. We overcame this hurdle by using a 96-well format, flow cytometry–based, high-throughout neutralization assay [27, 28]. We screened baseline and follow-up

samples from all enrolled children at 2 dilutions for the presence of neutralizing antibody before selecting samples for more-comprehensive neutralization testing (Supplementary Materials). Our study demonstrates that, while simple to perform, IgG ELISA and related assays are unreliable methods for detection of DENV infections when samples are collected 12 months apart, particularly for repeat infections. In repeat infections, while total DENV-specific IgG levels initially increased, over several months they declined back to baseline levels. In contrast, neutralizing antibody breadth appeared to be permanently changed after a repeat infection.

With respect to primary DENV infections, factors other than preexisting immunity, such as infecting DENV strain or serotype and/or intrinsic host factors, must influence development of inapparent versus apparent infection [30–33]. Here, we explored whether inapparent and apparent primary infections stimulated different antibody responses in children and found no differences in DENV-specific IgG or neutralization quality. Our data also demonstrated that the duration of time following primary DENV infections negatively correlated with the breadth of neutralization, suggesting that neutralizing antibody responses evolve from cross-neutralizing to serotype-specific responses by 8–12 months after infection. Others have also observed that primary DENV exposure results in cross-neutralizing/protective antibodies that gradually become monotypic as time elapses [34–36], although the mechanisms responsible for the waning of cross-neutralization following primary DENV infections have not been defined. We observed a strong association between the presence of DENV-specific IgM and cross-neutralization, indicating that IgM may, in part, be responsible for cross-neutralization. However, IgM cannot be the only explanation because the period of transient cross-neutralization extended to 8–12 months, which is well beyond the 3–6-month window after infection during which IgM is detectable.

For repeat apparent infections, we characterized antibody quantity and quality before, during, and after infection. Although we did not observe a significant rise in levels of DENV-specific IgG during the acute phase of infection, by 2–4 weeks after infection most children had elevated levels of IgG, compared with preinfection levels. Surprisingly, the elevation in IgG levels after repeat infection was not stable; in fact, when follow-up samples were collected several months after reinfection, IgG levels had declined to preinfection and acute-phase levels. During the acute phase, we observed boosting of neutralizing antibody response to the previously infecting serotype in most repeat infection cases. At 2–4 weeks after a repeat infection, children had high levels of cross-neutralizing antibodies. When follow-up samples were collected several months later, although the overall magnitude of neutralizing antibody had decreased, the breath of neutralizing antibody remained greater than in the sample obtained before repeat infection. We did not follow these children for >12 months, but we believe that these cross-neutralizing antibody responses are long-lived, perhaps for a lifetime. Thus, although, the quantity of DENV-specific IgG following a repeat DENV infection returned to preinfection and acute-phase levels, the quality of the neutralization response was stably altered.

Many studies have focused on how antibodies may enhance DENV infections and increase the risk of developing dengue hemorrhagic fever [10, 12–16]. While it is well documented that most DENV infections are clinically inapparent [3], factors responsible for inapparent infections have not been thoroughly investigated. Here, we observed a strong association between the breadth of neutralization and the manifestation of symptoms, by comparing the number of serotypes neutralized and quantifying the relative breadth of neutralization before repeat infections. We found that children with repeat inapparent infections had a greater number of broadly neutralizing preexisting DENV antibodies than children with apparent reinfections. This is perhaps our most interesting finding in that it demonstrates an association between the breadth of preexisting DENV neutralizing antibodies and the likelihood of developing an inapparent versus an apparent infection, thus suggesting an important role of preexisting neutralizing antibodies in protection from clinical symptom manifestation in repeat DENV infections.

There are different nonexclusive possible explanations for this association. Recent studies based on careful analysis of serial first, second, and third DENV infections in Nicaragua and models based on large dengue data sets from Thailand indicate that the period of cross-protection after a primary infection can be up to 2–3 years after infection [37, 38]. Children in our study who had secondary inapparent infections may have been more recently exposed to their first infection, compared with children who had secondary apparent infections. Our data showing the absence of IgM in baseline samples from nearly all children with repeat infections (both apparent and inapparent) demonstrates that these repeat cases did not occur within the first 6 months of the first infection. It remains possible that children who had secondary inapparent infections were still within the 1–3-year window of cross-protection, unlike children who had secondary apparent infections, who were well beyond this period of cross-protection.

Another possibility is that children experiencing repeat apparent infections were exposed to secondary infections, whereas most inapparent cases resulted from tertiary or quaternary (ie, postsecondary) exposure. Thus, the broadly neutralizing baseline responses may have been due to ≥2 previous DENV infections, whereas the children with monotypic responses may have only been exposed to a single serotype. Indeed, a recent study using cohort samples from Iquitos, Peru, demonstrated that individuals experiencing postsecondary infections were more likely to develop inapparent infections, compared with people with so-called true second infections [17]. It is worthwhile to note

that, although it is possible that children who appeared to have inapparent second infections were actually experiencing postsecondary infections, the degree of neutralization breadth we observed before infection in those cases was not significantly different from that for children with primary infections ($P = .3$; Table 2). It is likely that proximity to previous infection and number of previous infections, together with person-to-person variation, all influence whether someone will experience inapparent or apparent DENV reinfection.

Our study was not designed to explore questions about the role of antibodies in dengue hemorrhagic fever, which is typically seen in <5% of apparent dengue cases and 1% of dengue cases overall [39]. Even with a rising incidence of severe dengue in Sri Lanka [26], our cohort size of 799 children was too small to obtain the number of dengue hemorrhagic fever cases required for such analysis [8]. The role of antibodies in inapparent infections is a neglected topic and increasingly relevant as dengue vaccines enter clinical trials. Recent trial results of a highly anticipated DENV vaccine candidate signify the importance of understanding the role that preexisting DENV neutralizing antibodies play in protection from subsequent dengue disease and adverse clinical outcomes [40]. Our findings point to an important role for heterotypic neutralizing antibodies in protection from dengue disease. Further studies are needed to better define the protective properties of these cross-neutralizing antibodies.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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

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