ABSTRACT

PATEL, BHUMI. Dissecting the Human Serum Antibody Response to Repeat Dengue Virus Infections. (Under the direction of Dr. Aravinda de Silva).

Dengue virus (DENV) is a mosquito-borne flavivirus and the causative agent of dengue fever (DF) and dengue hemorrhagic fever (DHF). Several hundred million people are estimated to acquire DENV infections each year and it has become imperative that a vaccine is needed in order to reduce morbidity. As there are four serotypes of DENV, people can be infected multiple times, each time with a new serotype. Based on recent studies, the dengue field has learned a lot about the properties of neutralizing antibodies generated in people exposed to dengue for the first time (primary infections) but little is known regarding the immune response generated during a repeat infection with a new serotype (secondary infection). Following primary infections, people develop lifelong protective immunity against the serotype of infection. Primary infections stimulate serotype-cross reactive and serotype-specific (to the serotype of infection) antibodies. However, only a small fraction of serotype-specific antibodies that bind to quaternary structure epitopes are responsible for DENV neutralization. In the present study, we investigated the properties of neutralizing antibodies produced during a secondary infection. Following a secondary infection, people develop neutralizing/protective antibodies to multiple serotypes including serotypes that have not infected the individual. An antibody depletion technique using beads coated with purified virus was used to measure levels of serotype-specific and cross-reactive antibodies and their relative contribution to neutralization. We observed two types of responses: in some sera, dengue virus neutralization was dominated by cross-reactive antibodies, whereas in other sera both type-specific and cross-reactive antibodies contributed to neutralization. Thus, unlike primary sera, secondary sera
contain antibodies that cross-neutralize and presumably cross-protect from DENVs. Furthermore, depletion assays conducted with recombinant DENV protein revealed that these neutralizing cross-reactive antibodies recognize both simple epitopes preserved on the E (envelope) protein and more complex epitopes found on the intact virion. These results significant implications for the DENV Ab and vaccine field.
Dissecting the Human Serum Antibody Response to Repeat Dengue Virus Infections

by
Bhumi Patel

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APPROVED BY:

_______________________________  ______________________________
Dr. Scott Laster  Dr. Frank Scholle
Committee Chair

______________________________
Dr. Aravinda de Silva
Bhumi Patel grew up in Sanford, NC before moving to Chapel Hill, NC for her undergraduate training at the University of North Carolina at Chapel Hill. She completed her Bachelor of Science in Biology at UNC-CH after which she was admitted to North Carolina State University in the Immunology Program. Under the guidance of Dr. Aravinda de Silva at UNC, she completed her Master’s research concerning the analysis of the human antibody response following repeat dengue infections.
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Love you guys!
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LIST OF ABBREVIATIONS

Ab: Antibody
ADE: Antibody dependent enhancement
BSA: Bovine serum albumin
CR: Cross-reactive
DC-SIGN: Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
DENV: Dengue virus
DF: Dengue fever
DHF: Dengue hemorrhagic fever
E: Envelope glycoprotein
EBV: Epstein-barr virus
EDI-III: Envelope domain I-III
ELISA: Enzyme-linked immunosorbent assay
hMAbs: Human monoclonal antibodies
HMGB1: High-mobility group protein 1
M: Membrane protein
NS: Non-structural protein
OD: Optimal density
prM: Pre-membrane protein
rE: Recombinant envelope glycoprotein
TS: type-specific
1. INTRODUCTION

1.1 Overview

Dengue fever and Dengue hemorrhagic fever are diseases caused by the etiological agent, dengue virus (DENV), and are most prevalent in Asia and Latin America. DENV is a mosquito borne virus (Genus-Flavirus; Family-Flaviviridae) that is predominantly transmitted by the mosquito, *Aedes aegypti*. Other members of the Flaviviridae family include Yellow fever, West Nile and Japanese encephalitis and tick-borne encephalitis viruses. There are four closely related serotypes of dengue, named DENV1 through DENV4 [1]. Approximately 390 million people globally become infected with this virus each year, with approximately 85% of these infections being asymptomatic. Most symptomatic individuals develop dengue fever with symptoms of biphasic fever, muscle pain, joint pain, headache and nausea. For about 500,000 people a year, this febrile phase of the disease can progress to more severe forms of dengue which include dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) [2-4]. The risk of DHF is greater in people experiencing a secondary DENV infection compared to a primary DENV infection. The theory of antibody dependent enhancement (ADE), in which pre-existing dengue antibodies aid in viral infection of FcγR-bearing cells, has been proposed as an explanation for the increased risk of DHF following secondary infection [5]. Due to social and ecological changes in many parts of the world that promote transmission, dengue has become a reemerging pandemic. Vaccines offer one of the best strategies for preventing and controlling dengue. In order to formulate a vaccine that will provide durable protection against all four serotypes of DENV, a better understanding of the immune response following natural
DENV infections in humans is needed. The goal of this study is to tease apart the antibody response in humans following repeat DENV infections and gain an understanding of the properties of strongly neutralizing cross-reactive antibodies that provide protection against all four serotypes.

1.2 Clinical Disease

Following inoculation of the skin by a feeding mosquito, DENV infects and replicates in resident skin dendritic cells called Langerhans cells. These infected Langerhans cells then migrate from the skin and travel to the draining lymph node where the virus spreads to monocytes and macrophages [6]. Once in the lymphatic and circulatory system, the virus can spread to other parts of the body [7].

Dengue Fever

The most common symptomatic manifestation of DENV infection is DF. Once infected, there is an incubation period of approximately one week after which DF symptoms appear. This symptomatic phase of infection is divided into three phases, the febrile, critical and recovery phase. During classic DF, individuals move from the febrile phase to the recovery phase with little to no time in the critical phase [8]. During the febrile phase, symptoms include fever, headache, myalgias, abdominal pain, nausea and the appearance of a polymorphic rash that is most commonly seen on the trunk, the insides of the arms and plantar and palmar surfaces. DF is generally milder in children when compared to adults. Treatment of dengue fever includes liberal oral administration of fluids and antipyretic treatment [9].
**Dengue Hemorrhagic Fever and Dengue Shock Syndrome**

DHF and DSS are the more severe manifestations of DENV infection and result in a critical phase that is characterized by hemorrhage, increased vascular permeability with leakage of intravascular fluid into interstitial spaces and hepatomegaly [10]. Viremia in patients with DHF and DSS is generally higher than in patients with DF. This may be attributed to ADE by pre-existing sub-neutralizing populations of cross-reactive (binds to more than one serotype of dengue) antibodies. These more severe cases of dengue infection are more prevalent in children in endemic areas where more than one serotype of dengue is circulating [11]. The exact cause of the progression from DF to DHF or DSS is not completely understood. This is in part due to the lack of understanding of the underlying mechanisms of endothelial dysfunction that lead to the defining characteristic of vascular leakage during DHF and DSS. Soluble factors such as TNFα, IL-6, IL-8, IL-10, IL-12 and HMGB1 produced by various immune cells have been proposed to increase permeability in primary endothelial cells. In addition, complement activation by NS1 and anti-NS1 antibodies may also be responsible for induction of vascular leakage [12]. Currently, there is no therapeutic treatment and management of the disease involves supportive care such as fluid administration [9].

### 1.3 DENV Structure

Dengue virus is an enveloped positive-stranded RNA virus with icosahedral symmetry that consists of three structural and seven nonstructural proteins. The two main structural proteins found in the viral envelope include the envelope (E) and membrane (M) proteins. E protein mediates attachment and fusion of viral and cellular membranes. Each monomer of E
protein consists of three domains, EDI-III, and is the main target of neutralizing antibodies produced during dengue infections [13]. The viral fusion peptide is located on the tip of EDII and is highly conserved between serotypes [14]. In its native conformation on a virus particle, 180 E protein monomers form head-to-tail homodimers that lie flat and pack together in rafts on the surface of the viral membrane [15](Fig. 1). The prM protein is also a target of the immune system but elicits weakly neutralizing antibodies that may contribute to enhancement of heterotypic DENV infection [16].

Figure 1. DENV Envelope glycoprotein. E is the main surface exposed protein of DENV and is therefore the target of many antibodies. In its native conformation, E monomers form head to tail dimers that pack together on the surface of the virus particle. E protein is composed of three domains, EDI, EDII and EDIII, with the fusion peptide located on the tip of ED II. Image taken from Kuhn, RJ et al, Cell, 2002 [15].
1.4 DENV Cell Biology

Dengue virus enters the host cell through receptor-mediated endocytosis. The exact receptor that mediates this internalization is unknown but the receptor binding site on the virus is thought to be located in ED III [13]. In vitro, attachment factors such as DC-SIGN (dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin) have been identified that aid in viral infection [17]. Following entry, the pH of the endosome acidifies and the virus undergoes a conformational change in which the 90 E protein dimers rearrange to form 60 trimers. This rearrangement exposes the fusion peptide and leads to fusion of the viral membrane with the endosomal membrane of the host. Once the viral RNA is released into the host cytoplasm, host translational machinery is used to produce the different viral components. Assembly of the virion takes place on the endoplasmic reticulum (ER) of the host cell. Initially, the virions are immature with trimers of E/prM heterodimers forming spikes on the surface of the virion. As the virus particles progress through the secretory pathway, the host pH-dependent protease, furin, cleaves the prM protein to produce M protein [18](Fig. 2). This mature version of the virus has a smooth surface with E homodimers lying flat on the surface of the viral membrane. This cleavage event during maturation of the virus is inefficient and results in the release of both mature, immature and partially mature virus in cell-culture derived DENV [19](Fig. 3).
**Figure 2. Life cycle of DENV.** The DENV particle binds to its receptor on the host cell and undergoes endocytosis. As the pH of the endosome acidifies, the virus undergoes a conformational change and fuses with the host endosomal membrane and releases its genome into the host cytoplasm. Viral proteins are then translated using host machinery and DENV particles are assembled in the endoplasmic reticulum. The virus particles undergo post-translational modification in the Golgi. Once in the secretory pathway, the pH-dependent host protease, furin, cleaves prM on the surface of the protein to produce M protein. Cleavage of prM is inefficient and the particles exocytosed include immature, partially immature/mature and mature DENV particles. Image taken from Mukhopadhyay S. et al, Nature Reviews Microbiology 2005 [18].
Figure 3. DENV maturity. In the immature conformation of the virus before cleavage with the host protease, furin, prM and E dimers form trimers that spike up from the surface of the virion. After cleavage or prM, E dimers lie flat on the surface of the viral membrane and give the virus a smooth appearance. Image taken from Perera R. et al, Current Opinion in Microbiology 2008 [20].

1.5 Human Response to DENV Infection

Primary Infection

Most symptomatic dengue infections result in DF with an incubation period of approximately 4 to 7 days followed by the appearance of classic DF symptoms such as fever, headache, myalgias, abdominal pain and nausea [21]. Following primary infections with dengue, people develop life-long immunity against the serotype of infection (homologous serotype) but not against the other three serotypes (heterologous serotypes). IgM levels peak
at around two weeks following onset of illness and wane to undetectable levels by 2-3 months. IgG antibodies are detected 2 weeks after the onset of illness and are responsible for life-long protection against the homologous serotype. This IgG response of mainly IgG1 and IgG3 is highly cross-reactive with all four serotypes of DENV with only a small portion responsible for type-specific neutralization of the homologous serotype [22]. Although a primary infection results in life-long protection against only the homologous serotype, there is a short period of several months following the infection in which there is also protection against heterologous serotypes [23, 24].

Secondary Infection

Since primary infection results in protection against only the serotype of infection, individuals are susceptible to secondary infections with a heterologous serotype. During secondary DENV infections, stimulation of memory B cells formed during primary infection results in a stronger and more rapid antibody response that is skewed towards the serotype of the primary infection [25]. This antibody response broadens and encompasses protection not only against the serotype of the primary and secondary infection but also other serotypes the individual has never been exposed to. One of the unique attributes of dengue is that people experiencing secondary infections with a new serotype are at a greater risk of developing more serious conditions such as DHF and DSS. The leading theory supporting this higher risk is antibody dependent enhancement (ADE). During ADE, cross-reactive, weakly neutralizing antibodies formed during primary infection enhance secondary infection by aiding in viral entry via FcγR on monocytes and macrophages [26].
**Figure 4. Antibody Dependent Enhancement of DENV.** When the concentration of DENV antibodies is below a protective threshold, the antibodies can bind to DENV and aid in entry of the virus into host cells via FcγRs on monocytes and macrophages. This results in increased viral load and suppression of the anti-viral response. Image taken and modified from Murphy B.R. et al, Annual Review of Immunology, 2011 [27].

### 1.6 DENV Epitopes Recognized by Human Antibodies

Following DENV infection, individuals develop antibodies to the structural proteins, E, prM and Capsid (C), as well as the nonstructural proteins, NS1, NS3 and NS5 [28]. Since the E protein is the most surface exposed protein on DENV particles, it is the main target of antibodies generated following DENV infection [29]. Many of the antibodies generated during an infection are cross-reactive and target the fusion loop of the E protein. However, these antibodies are unlikely to provide protection against the homologous serotype during primary infection since the fusion loop is highly conserved between serotypes [30]. These neutralizing
antibodies must recognize an epitope that is not highly conserved between serotypes. Studies involving the use of mouse models to study the immune response have shown that the major target of type-specific neutralizing antibodies during primary infections is EDIII [31, 32]. However, studies with human immune sera in which EDIII binding antibodies were removed, showed that although there are neutralizing antibodies that recognize EDIII, they make up a small portion of the total population of neutralizing antibodies [33]. These studies make it clear that the immune response generated in mouse models following dengue infection differs from the response generated in humans following natural dengue infection.

Many groups have begun to use human monoclonal antibodies (hMAbs) to further elucidate DENV epitopes recognized by antibodies. These hMAbs are generated by transforming memory B cells from DENV immune individuals with EBV [34, 35]. Studies have shown that many of the epitopes these type-specific neutralizing hMAbs recognize are quaternary epitopes that are present on the intact virion but not on individual E monomers. More specifically, many of these antibodies target the EDI-II hinge region. In order for these antibodies to bind and neutralize DENV, E monomers must be present in specific arrangement that is only found on a whole virion particle. These results were also seen with primary dengue human immune sera. When depleted with whole virion particles of the homologous serotype, immune sera lose neutralizing activity to the homologous serotype. However, when depleted with recombinant E protein of the homologous serotype, neutralizing activity of the immune sera remains unaffected [36]. These studies support the idea that the main epitopes type-specific neutralizing antibodies recognize in primary human immune sera are quaternary epitopes that are only preserved on the intact DENV particle.
1.7 Prospective Vaccines

The development of an effective DENV vaccine poses to be a particularly challenging task due to the presence of four different serotypes of DENV and the ability of the immune system to enhance infection through ADE. The goal of an ideal vaccine would be to provide life-long protection against all four serotypes of dengue and to provide a robust enough immune response that would overcome the problem of ADE. If the amount of protective antibodies wane below a certain level, these antibodies could enhance infection and exacerbate disease. Current efforts to develop a DENV vaccine have focused on live attenuated virus vaccines, inactivated virus vaccines and subunit virus vaccines.

Currently the leading candidates for DENV vaccines are live attenuated vaccines. These vaccines have many requirements that must be filled in order to be effective. The level of viremia induced in individuals should be low enough to restrict the development of symptoms and reduce transmissibility to mosquitoes. However, the virus must be highly infective in low doses and be able to replicate efficiently in tissue culture in order to be economically feasible [37]. The leading live attenuated vaccine has been ChimeriVax which contained prM and E protein genes from each of the four DENV serotypes placed into the YF17D (yellow fever) vaccine strain. Four chimeric monovalent viruses were produced and the study progressed through phase IIB clinical trials. However, the chimeric virus vaccine failed with only 30% efficacy against natural DENV infections [38]. Despite this set back, there are several other vaccine candidates clinical trials.
1.8 Objective

With several hundred million people estimated to acquire DENV infections each year, it is becoming increasingly apparent that a better understanding of dengue neutralization in natural human infections is needed [2]. Recent developments in the formulation of a dengue vaccine have made it clear that the immune status of an individual prior to vaccination greatly affects the type of response elicited post vaccination [39, 40]. By further studying the antibody response generated following both primary and repeat natural dengue infections, we can apply this knowledge to potential vaccine candidates. The aim of this study is to further characterize the antibody populations generated following natural repeat infections of DENV.

After primary DENV infection, people develop a mix of DENV serotype cross-reactive and type-specific antibodies. The cross-reactive antibodies are weakly neutralizing and have been implicated in antibody dependent enhancement of DENVs during secondary infections [36, 41-43]. The serotype-specific antibodies are strongly neutralizing and presumably responsible for long-term protection against re-infection with the same serotype. Quaternary epitopes formed after assembly of E molecules into higher order structures required for virion assembly are the dominant targets of type-specific neutralizing antibodies [36, 44, 45]. While this type-specific population of antibodies dominates the response following primary infections of DENV, little is known about the populations of antibodies produced following repeat infections of DENV. Recent studies have recognized a population of human broadly neutralizing DENV antibodies produced by plasmablasts soon after secondary infection [46, 47]. However, it remains to be identified if this population of antibodies persists years after
infection. In the present study we investigated the properties of serum neutralizing antibodies produced after recovery from repeat DENV infections using an antibody depletion technique. We found that people exposed to repeat infections had distinct populations of type-specific and cross-reactive neutralizing antibodies and the proportion of each type of antibodies varied between individuals. The cross-neutralizing antibodies recognized simple epitopes present on E protein and more complex epitopes that were only displayed on intact virions.
2. MATERIALS AND METHODS

2.1 Immune Sera

Human sera were collected from individuals who had experienced a DENV infection during travel to a DENV endemic area (Table 1). All donations were collected in compliance with the Institutional Review Board of the University of North Carolina at Chapel Hill (Protocol #08-0895). Samples were classified as primary infections if they had neutralizing antibody to only one serotype (≥ 1:40 Neut50 titers) or a titer that is 4-fold higher for one DENV serotype compared to low titers (Netu50 ≤ 1:40) to the other serotypes. Samples were classified as secondary infections if they had Neut50 titers greater than a 1:40 dilution for two or more DENV serotypes.

2.2 Virus and Recombinant Envelope Protein

WHO reference strains, DENV1 (West Pac 74), DENV2 (S-16803), DENV3 (CH-53489) and DENV4 (TVP-376) were used in the present study. The strains were grown in C6/36 mosquito cells to generate infectious stocks and Vero-81 mammalian cells to generate purified antigen. DENVs from culture media were purified by density gradient and ultracentrifugation as previously described [33]. DENV2 purified antigen was purchased from Microbix Biosystems, Inc. (Mississauga, Ontario, Canada). Dengue recombinant envelope protein was produced by the UNC Protein Expression and Purification Core Facility using Bac-to-Bac Baculovirus Expression System by Invitrogen Life Technologies with Sf9 cells. The amino acid residues for envelope protein construct was DENV2 E80% (1-397). Structure of the recombinant
proteins were verified by conducting ELISAs with monoclonal antibodies that target various regions of the envelope protein in its native conformation on the DENV particle.

2.3 Whole virus depletion of DENV-Specific Antibodies from Human DENV Immune Sera

Purified DENV was absorbed onto 4.5-μm Polybead polystyrene microspheres (Polysciences, Inc., Cat.# 17135-5) at a bead (μl) to ligand (μg) ratio of 5:2. Beads were washed three times with 0.1M Borate buffer (pH 8.5) followed by an overnight incubation with purified DENV and 0.1M Borate buffer (8.5) at room temperature (RT). Control beads were incubated with the equivalent amount of BSA. The control and virus absorbed beads were then blocked with a 10 mg/ml BSA solution for 30 minutes at RT three times followed by four washes with PBS. DENV-specific antibodies were depleted from human sera by incubating virus absorbed beads with human sera diluted 1:10 in PBS for 45 minutes at 37°C three times with end-over-end mixing. Successful depletion of DENV-specific antibodies was confirmed via an ELISA with purified DENV coated plates.

2.4 Recombinant E protein depletion of DENV-specific Antibodies from Human DENV Immune Sera

Purified rE protein was conjugated to magnetic dynabeads M-270 Epoxy (Invitrogen by Life Technologies, Cat. # 14302D) with a bead (mg) to ligand (μg) ratio of 5:1. Beads were washed three times with 0.1M Sodium Phosphate (7.4) followed by an overnight incubation with equal volumes of purified rE protein, 0.1M Borate buffer (pH 13) and 3M Ammonium sulfate at
37°C. Control beads were incubated with the equivalent amount of BSA. Control and rE conjugated beads were then blocked by washing with a 10 mg/ml BSA solution four times followed by four washes with PBS. DENV rE-specific antibodies were depleted from human sera by incubating rE conjugated beads with human sera diluted 1:10 in PBS for 45 minutes at 37°C three times with end-over-end mixing. Successful depletion of DENV rE-specific antibodies was confirmed via an ELISA with purified rE coated plates.

2.5 Detection of DENV Binding Antibodies via ELISA

ELISAs were conducted for both confirmation of depletion and the assessment of binding activity to all four DENV serotypes following depletion of human DENV immune sera. Plates were coated with either 50 ng of purified DENV or 100 ng of DENV rE in carbonate buffer at pH 9.6 for 2 hours at room temperature. The plates were blocked with Tris-buffered Saline containing 0.05% Tween 20 with 3% Normal Goat Serum followed by an incubation with a 1:40 dilution of control or DENV depleted human sera for 1 hour at 37°C. Alkaline phosphatase conjugated Goat anti-human IgG (Sigma) was added to the plates for 1 hour at 37°C. Finally, p-nitrophenyl phosphate substrate was added the plates and the reaction was allowed to proceed until a sufficient O.D. (optical density) signal developed. The signal was measured using a spectrophotometer at an optical density of 405 nm.

2.6 DENV FACS-based Neutralization Assay

DENV neutralizing activity of human immune sera was assessed using a flow cytometry-based assay with U937 human monocytic cell line stably transfected with DC-SIGN as previously
described [48]. Serially diluted human sera were incubated with virus for 45 min at 37°C followed by the addition of U937 DC-SIGN cells. Cells were incubated with virus for 2 hours at 37°C, washed with media to remove immune sera and unbound virus and incubated for 24 hours at 37°C. Cells were fixed, permeabilized and stained with 2H2-Alexa Flour 488, a mouse monoclonal that binds to DENV pre-membrane protein. Infected cells were quantified using a Guava flow-cytometer (Milipore). Stained cells were analyzed to calculate 50% neutralization titers as previously described [49].
3. RESULTS

3.1 Depletion of DENV binding antibodies from primary DENV2 immune sera

We used a panel of eight human DENV immune sera from people who had been exposed to DENV during travel or previous residency in a dengue endemic area (Table 1). All the samples were collected between 9 and 30 years of their most likely exposure to DENV. Three of these sera are from primary DENV2 cases while the rest are from people exposed to two or more infections as they had high levels of neutralizing antibodies to two or more serotypes. A serum sample from a dengue naïve human donor was used as a negative control.

To determine if DENV serotype cross-reactive or type-specific antibodies were responsible for neutralizing activity in each serum sample, we used polystyrene beads coated with purified dengue virions to deplete different populations of antibodies from serum samples. The antibodies in each serum sample were depleted using beads coated with purified DENV2 or an equal mixture of DENV1, 3 and 4. As a control, the serum was also incubated with beads coated with BSA. The DENV2 depletions were expected to remove DENV2 type-specific and DENV serotype cross-reactive antibodies, while retaining any DENV1, 3 or 4 type-specific antibodies in the sample. After depleting with DENV2, any reduction in DENV1, 3 and 4 neutralizing activity in the sample was attributed to cross-reactive antibodies. Similarly, when serum samples were depleted with beads containing equal amounts of purified DENV1, 3 and 4, any reduction in DENV2 neutralizing antibody was attributed to cross-reactive antibodies.
Table 1. Panel of naïve and late convalescent DENV-immune human sera

<table>
<thead>
<tr>
<th>Donor ID</th>
<th>Location of infection</th>
<th>Year of infection</th>
<th>Interval between infection and time of collection (years)</th>
<th>Reciprocal of Neut$_{50}$ Titer</th>
<th>Sero Status$^\dagger$</th>
</tr>
</thead>
<tbody>
<tr>
<td>030</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>43 47 53 46</td>
<td>Naïve</td>
</tr>
<tr>
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<td>1998</td>
<td>10</td>
<td>205 1280 153 96</td>
<td>Primary DENV2</td>
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<tr>
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<td>30</td>
<td>304 630 452 105</td>
<td>Secondary</td>
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<td>25</td>
<td>179 233 47 71</td>
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<td>25</td>
<td>878 249 218 71</td>
<td>Secondary</td>
</tr>
<tr>
<td>121</td>
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<td>1995</td>
<td>16</td>
<td>92 371 325 179</td>
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<tr>
<td>130</td>
<td>French Polynesia</td>
<td>1989</td>
<td>25</td>
<td>674 347 86 62</td>
<td>Secondary</td>
</tr>
</tbody>
</table>

$^\dagger$ Primary defined as reciprocal Neut$_{50}$ titer greater than 40 for one serotype or a reciprocal Neut$_{50}$ titer that is at least 4-fold greater for one serotype. Secondary defined as reciprocal Neut$_{50}$ titer greater than 40 for two or more serotypes.
Depletion of two primary DENV2-immune sera using beads coated with DENV2 resulted in a reduction of binding to DENV2 as well as DENV1, 3 and 4, demonstrating removal of cross-reactive and type-specific antibodies in the sample (Fig. 5 C and F). Antibody depletion with the homologous virus antigen, led to a large drop in serotype 2 neutralization (Fig. 5 A, B, D and E). Following removal of DENV1, 3 and 4 binding antibodies, ELISA confirmed removal of cross-reactive antibodies, while still retaining DENV2 type-specific antibodies in the sample (Fig. 5 I and L). The removal of cross-reactive antibodies did not reduce the potency of neutralizing antibodies in the sample establishing that type-specific antibodies were responsible for the neutralization activity in these primary sera (Fig. 5G, H, J and K). As we have previously shown, this data demonstrates that following primary infection neutralization of the homologous serotype (the serotype of infection) is mediated by type-specific antibodies with negligible contribution by cross-reactive antibodies [36].

3.2 Depletion of DENV binding antibodies from secondary DENV immune sera

Following secondary/repeat infections, individuals are typically protected from two or more serotypes, however it is unknown whether individuals generate individual type-specific neutralizing populations of antibodies against each serotype or a broadly neutralizing cross-reactive population against all of the serotypes. We depleted repeat infection sera using beads coated with DENV2 or a mixture of DENV1, 3 and 4. Similar to the depletion studies previously conducted with primary sera, depletion of DENV2 binding antibodies from secondary sera resulted in a reduction of binding to all four serotypes of DENV with varying levels of DENV1, 3 and 4 type-specific antibodies remaining in the samples (Fig. 6 C and F).
Figure 5. Binding and neutralization properties of primary DENV-immune human sera depleted of DENV-specific antibodies. Polystyrene beads coated with either purified DENV2 or a mixture of purified DENV1, 3 and 4 were used to deplete DENV2 primary immune sera (DT001 and DT110) of DENV-specific antibodies. Following depletion of DENV2-binding antibodies, sera was tested for binding (C and F) and neutralization (A, B,D and E) of DENV1-4. Sera depleted with the homologous serotype (DENV2) resulted in a reduction of binding to all four DENV's and a loss of neutralization of DENV2. Primary DENV2 immune sera were depleted of cross-reactive antibodies by using beads coated with DENV1, 3 and 4 and then tested for binding (I and L) and neutralization (G,H,J and K) of DENV1-4. Sera depleted of cross-reactive antibodies displayed a reduction in binding to DENV1, 3 and 4 but retained a population of type-specific antibodies that bound virus of the homologous serotype (DENV2). Neutralization of the homologous serotype (DENV2) following depletion of cross-reactive antibodies, remained unaffected when compared to control depleted serum.
Similarly, when the samples were depleted using a mixture of DENV1, 3 and 4 antigens, cross-reactive antibodies were removed, while retaining varying levels of DENV2 type-specific antibodies in the samples (Fig. 6 I and L). The contribution of the depleted cross-reactive antibodies to neutralization of all four serotypes of DENV varied from sample to sample. Within our panel of secondary/repeat infection sera, we observed two patterns of neutralization. In some samples, populations of serotype cross-reactive antibodies accounted for all the neutralization activity against the different serotypes. Other samples had a mix of type-specific and cross-reactive antibodies contributing to neutralization.

DT000, a secondary DENV-immune human serum sample that exhibits moderate neutralization of all four DENV serotypes, was a case in which neutralization is primarily mediated by cross-reactive antibodies (Table 1). Following depletion of DENV2 binding antibodies, there was significant loss of DENV2 neutralization as expected. However, there was also a loss of neutralization to DENV1, 3 and 4 (Fig. 6A and B). These results demonstrate that neutralization of DENV1, 3 and 4 is mediated by cross-reactive antibodies. Reciprocal depletion with DENV1, 3 and 4, resulted in a loss of neutralization of DENV2, indicating that a cross-reactive population of antibodies is responsible for neutralization of DENV2 (Fig. 6 G and H). Collectively, these results demonstrated that for the secondary sample, DT000, cross-reactive antibodies dominate neutralization of all four DENV serotypes. Two of the secondary DENV immune sera from our panel, DT000 and DT121, displayed this broadly cross-neutralizing pattern, however the remaining three, DT025, DT027 and DT130, displayed a different neutralization pattern (Table 2)(Fig. 8).
DT130, a secondary DENV-immune human serum that moderately neutralizes DENV1 and DENV2, is a case in which both type-specific and cross-reactive antibodies play a role in DENV neutralization. Following removal of DENV2 binding antibodies, there was a loss of neutralization of DENV2 as expected. However, unlike DT000, neutralization of DENV1 remained relatively unaffected when compared to control depleted sera (Fig. 6 D and E). This result indicates that a type-specific population of antibodies is responsible for DENV1 neutralization. Reciprocal depletion with DEN1, 3 and 4, resulted in loss of neutralization of DENV2 suggesting that neutralization of DENV2 is mediated by a population of cross-reactive antibodies. Overall, neutralization of DENV by DT130 is dominated by a type-specific population for DENV1 and a cross-reactive population for DENV2. Samples DT025 and DT027 also exhibited this familiar phenotype in which cross-reactive populations of antibodies were responsible for neutralization of DENV2, 3 and 4, while a type-specific population was responsible for neutralization of DENV1 (Table 2)(Fig. 8). Overall, unlike primary DENV infections that stimulate neutralizing antibodies that bind to epitopes that are unique to each serotypes, following repeat infections people develop neutralizing antibodies that recognize type-specific or conserved epitopes. The proportions of these two classes of antibodies vary between individuals exposed to repeat infections.
Figure 6. Binding and neutralization properties of secondary DENV-immune human sera depleted of DENV-specific antibodies. Polystyrene beads coated with either purified DENV2 or a mixture of purified DENV1, 3 and 4 were used to deplete secondary DENV-immune sera (DT000 and DT130) of DENV-specific antibodies. Following depletion of DENV2-binding antibodies, sera was tested for binding (C and F) and neutralization (A, B,D and E) of DENV1-4. Depletion of DENV2 binding antibodies from the secondary immune serum, DT 000, resulted in a reduction of binding and a loss of neutralization of all four DENV’s. In contrast, depletion of DENV2 binding antibodies from the secondary immune serum, DT130, resulted in a reduction of binding to DENV2, 3 and 4 with a substantial type-specific population of antibodies remaining for DENV1. Accordingly, depletion of DENV2 binding antibodies resulted in a loss of neutralization of DENV2 with little effect on the neutralization of DENV1. Reciprocal depletions of secondary DENV-immune sera were conducted using beads coated with DENV1, 3 and 4. Depleted sera were tested for binding (I and L) and neutralization (G,H,J and K) of DENV1-4. Both secondary samples, DT000 and DT130 exhibited a loss of binding and neutralization of all four DENV’s following depletion of DENV1, 3 and 4 binding antibodies.
3.3 Neutralizing activity of secondary sera following depletion DENV2 rE binding antibodies

Our previous studies with primary DENV immune sera revealed that type-specific neutralizing antibodies mostly targeted quaternary epitopes expressed on E protein dimers or higher order structures but not recombinant E protein (rE), which is mainly a monomer in solution[36]. We next, addressed if cross-reactive neutralizing antibodies induced by repeat DENV infections targeted simple or quaternary epitopes on E protein. DENV2 rE covalently coupled to dynabeads was used to deplete primary and secondary DENV immune sera of DENV2 rE binding antibodies. The depleted sera were tested to determine whether the neutralizing antibodies recognize an epitope preserved on rE or the whole virion particle. As previously reported by us, primary DENV2 immune sera depleted of DENV2 rE binding antibodies resulted in no loss of neutralization potency indicating that these type-specific DENV2 neutralizing antibodies target an epitope preserved on the whole virion particle (Fig. 7 A, B, D and E). Depletion of secondary DENV immune sera of DENV2 rE binding antibodies resulted in varying effects on binding and neutralization of DENVs.

Removal of DENV2 rE binding antibodies from DT000 resulted in loss of neutralization of DENV1, 2 and 4 but not of DENV3 (Fig. 7G and H). Combined with our results from the whole virus depletion of DENV2 binding antibodies, these results indicate that the cross-reactive and cross-neutralizing antibodies in this sample is a mixture of antibodies that bind to a conserved epitope on rE and a more complex conserved epitope on the intact virion.
DT130, a secondary sera composed of both type-specific (DENV1) and cross-reactive (DENV2) neutralizing antibodies, showed a different phenotype following depletion. Removal of DENV2 rE binding antibodies from DT130 resulted in a loss of neutralization of DENV2 but not of DENV1, demonstrating that the cross reactive DENV2 neutralizing antibodies bound to a simple epitope on the ectodomain of rE (Fig. 7 J and K).

Additionally, the depletion of DENV2 rE binding antibodies from DT025, 027 and 121 displayed variation in loss of neutralization between serotypes. Neutralization by type-specific DENV1 populations of antibodies remained relatively unaffected following DENV2 rE depletion as expected. However, neutralization mediated by cross-reactive antibodies following DENV2 rE depletion exhibited varying degrees of reduction (Table 2)(Fig. 8). Collectively, the results from the depletion of DENV2 rE binding antibodies from secondary sera demonstrates that cross-reactive neutralizing antibodies following secondary infection recognize epitopes preserved on rE protein as well as epitopes preserved on the whole DENV particle.
Figure 7. Binding and neutralization properties of primary and repeat/secondary DENV-immune human sera following depletion of DENV2 rE-binding antibodies. DENV2 rE was coupled covalently to dynabeads and incubated with DENV-immune sera to remove all DENV2 rE-binding antibodies. Following depletion, sera was assessed for binding (primary-C, F, secondary-I and L) and neutralization (primary- A, B, D and E, secondary- G, H, J and K) of DENV1-4. Removal of DENV2 rE-binding antibodies from the primary immune sera, DT001 and DT110, had little effect on binding to DENV1-4 and resulted in a modest reduction in neutralization of the homologous serotype (DENV2). Depletion of DENV2 rE-binding antibodies from the secondary immune sera, DT000 and DT130, also had little effect on binding to DENV1-4. However, neutralization of DENV1-4 following depletion varied between sera with DT000 retaining neutralization to DENV3 and DT130 retaining neutralization to DENV1.
Table 2. DENV serotype neutralization titers of primary and secondary immune sera depleted of DENV2 binding Abs, DENV1, 3 and 4 binding Abs and DENV2 rE binding Abs

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Figure 8. Binding properties of repeat/secondary DENV-immune human sera following depletion of DENV and DENV rE binding antibodies. Purified DENV or DENV rE was coupled to polystyrene beads or dynabeads, respectively, and incubated with DENV-immune sera to remove all DENV/DENV2 rE binding antibodies. Following depletion, the sera were assessed for binding (A, B and C).
4. DISCUSSION

It is known that following infection with dengue, humans develop neutralizing antibodies that confer protection against subsequent infections. Following primary infections this protection is limited to the serotype of infection. However, following secondary infection, this protection can extend to serotypes the person has not even seen. From previous studies, we have gained insight into the properties of neutralizing antibodies generated following primary infection and a more clear understanding of the epitopes these antibodies recognize on the virus particle. Both cross-reactive and type-specific antibodies are generated following a primary infection however, type-specific antibodies are responsible for protection against the homologous serotype. The majority of these type-specific antibodies recognize complex quaternary epitopes that are present on the intact virion particle.

Here, the results from a panel of five secondary/repeat DENV-immune sera demonstrates that unlike primary DENV-immune sera, the majority of neutralizing antibodies in secondary cases bind to epitopes that are conserved between serotypes. In some cases cross-reactive antibodies dominate the response, whereas in other cases both cross reactive and type-specific neutralizing antibodies co-circulate. Furthermore, we report that these cross-reactive neutralizing antibodies generated following secondary DENV infection recognize simple epitopes on soluble E protein as well as complex epitopes found only on the intact DENV particle.

The emergence of this population of cross-reactive neutralizing antibodies following a secondary DENV infection may be due to the affinity maturation of cross-reactive memory B cells generated following primary DENV infection[50]. If this were not the case, secondary
DENV infection would result in individual type-specific neutralizing populations of antibodies towards each serotype instead of a cross-reactive population as seen in DT000 and DT121. This supports the idea that the immune status of an individual following a primary DENV infection affects the type of antibodies the individual produces following a subsequent secondary DENV infection with another serotype. However, the existence of type-specific neutralizing antibodies in secondary sera, such as DT130 and DT027, hints that infection history may also play a role in how broadly this population of cross-reactive antibodies can neutralize across the four serotypes. In sera that exhibited broad neutralization of all four serotypes, such as DT000 and DT121, neutralization was dominated by cross-reactive antibodies. However, in sera that exhibited neutralization of only two serotypes, such as DT130 and DT027, neutralization of one serotype was dominated by cross-reactive antibodies while neutralization of the other serotype was dominated by type-specific antibodies. This difference in neutralization capacity of antibody populations may be due to secondary versus tertiary DENV infections. Since the infection history of these donors is unknown, we cannot readily make conclusions. However, it is probable that DT130 and DT027 are true secondary DENV infections while DT000 and DT121 are repeat infections with more than two serotypes. In agreement with this interpretation, our results suggest that with each successive DENV infection, the breadth of neutralization by cross-reactive antibodies increases [51].

Further support of this model of affinity maturation of cross-reactive antibodies from primary infections is provided by studies involving the comparison of avidity and neutralization of both monoclonal antibodies and polyclonal sera following primary and secondary DENV infections. Analysis of polyclonal human sera following DENV infections
revealed that the avidity of DENV antibodies following secondary infection was higher than that of antibodies generated following a primary infection [52]. In agreement with this, studies focusing on group-reactive MAbs derived from primary DENV infected and secondary DENV infected patients found that the group-reactive MAbs from patients with secondary infection had stronger neutralization potencies and higher binding avidities than those derived from patients with primary infection [53]. Additional studies have identified a class of broadly neutralizing human antibodies produced by plasmablasts in hospitalized cases of secondary DENV infections. Structural analysis of these broadly neutralizing antibodies in complex with rE revealed that these antibodies recognize serotype invariant sites at the E dimer interface. Whether this population of antibody persists years after infection, remains to be answered. Collectively, these studies support the idea that weakly neutralizing antibodies generated followed primary DENV infection become potently neutralizing Abs after secondary DENV infection.

There are several limitations to this study. Infection history of the patients prior to collection remains unknown, therefore, definite conclusions relating antibody population characteristics to the number of repeat infections cannot be made. Secondly, our sample size is small. Thirdly, it remains in question whether the in vitro neutralization assay is an accurate indicator of protection in humans. By using a larger panel of samples with known infection history and less involved procedures, we can resolve these limitations and gain a better understanding of the change in antibody response with each repeat infection. Additionally, antibody sequencing can aid in detection of somatic mutations and reveal if neutralizing
antibodies following repeat infections are in fact antibodies generated during previous infections.

Our studies show that the antibody response following a repeat infection is very different from the antibody response following a primary infection. Unlike primary infection, repeat infections of DENV generate populations of cross-reactive antibodies that provide protection against all four serotypes. Furthermore, our studies provide support of the idea that the immune status of an individual as a primary vs. repeat infection, affects the breadth of neutralization of cross-reactive antibodies. These findings are relevant to the development of dengue vaccines that strive to provide protection against all four serotypes. Naïve individuals receiving a vaccine may generate more of a type-specific response similar to that of a primary infection. In the case of tetravalent vaccine formulations, these responses may not be balanced towards each serotype [39, 54]. On the other hand, DENV-immune individuals receiving a vaccine may generate a more effective broadly neutralizing response against all four serotypes as seen by the high efficacy of the Sanofi Pasteur CYD tetravalent DENV vaccine in pre-immune individuals when compared to the low efficacy in DENV naïve individuals [40]. Overall, it is clear that a better understanding of the antibody response transition from primary to repeat infection is needed in order to determine if certain vaccine formulations will induce a protective response against all four serotypes of DENV.
5. REFERENCES


