CHARACTERIZATION OF HUMAN ANTIBODIES AGAINST ENVELOPE PROTEIN OF DENGUE VIRUS DURING NATURAL INFECTION

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ABSTRACT

Despite decades of research to develop vaccines against dengue virus (DENV), there is no licensed dengue vaccine available, partly due to the lack of understanding of immune correlates of protection during natural infection. While neutralizing antibodies (Abs) against the envelope (E) protein of DENV are known to be protective, the role of different categories of anti-E Abs in protection and their epitopes remain unknown.

The long-term goal of this study is to facilitate the development of a safe and effective dengue vaccine. The objective of the study is to elucidate the categories and epitopes of human anti-E Abs that account for neutralization during natural DENV infection. We hypothesize that after primary DENV infection, type-specific (TS) anti-E Abs are responsible for neutralization against the infecting serotype; after a secondary DENV infection, group-reactive (GR) anti-E Abs, the major component of cross-reactive Abs, correlate with neutralization against the non-exposed serotypes.

In the first aim, we defined the role of TS anti-E Abs after primary DENV infection. We demonstrated that TS, rather than cross-reactive, anti-E Abs were responsible for the neutralizing activity and recognized structurally complex epitopes present on the virus particles rather than solubilized E protein. Moreover, we found that domain III of the E protein, though not the predominant epitope, was recognized by TS neutralizing Abs.
In the second aim, we investigated the role of GR anti-E Abs in the neutralizing activity against non-exposed serotypes after secondary DENV infection. We demonstrated that the GR monoclonal antibodies derived from individuals with secondary DENV infection had stronger binding avidity and neutralization potency than those from individuals with primary infection, and blocked DENV at post-attachment step. This suggested that dengue immune status of the host (primary versus secondary DENV infection) affects the quality of cross-reactive Abs. In addition, we found that in the sera of 29 patients with secondary DENV infection from a Nicaraguan cohort, the concentration of anti-fusion loop Abs, which represents GR anti-E Abs, did not correlate with neutralization titers against the current infecting DENV serotype but correlated with neutralization titers against the non-exposed serotypes.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Abs</td>
<td>antibodies</td>
</tr>
<tr>
<td>ADE</td>
<td>antibody-dependent enhancement</td>
</tr>
<tr>
<td>C</td>
<td>capsid</td>
</tr>
<tr>
<td>CR</td>
<td>complex-reactive</td>
</tr>
<tr>
<td>DENV</td>
<td>dengue virus</td>
</tr>
<tr>
<td>DF</td>
<td>dengue fever</td>
</tr>
<tr>
<td>DHF</td>
<td>dengue hemorrhagic fever</td>
</tr>
<tr>
<td>DI, II, III</td>
<td>domain I, II, III</td>
</tr>
<tr>
<td>DSS</td>
<td>dengue shock syndrome</td>
</tr>
<tr>
<td>E</td>
<td>envelope</td>
</tr>
<tr>
<td>FFU</td>
<td>focus forming unit</td>
</tr>
<tr>
<td>FL</td>
<td>fusion loop</td>
</tr>
<tr>
<td>FRNT</td>
<td>focus reduction neutralization test</td>
</tr>
<tr>
<td>GR</td>
<td>flavivirus group-reactive</td>
</tr>
<tr>
<td>HLA</td>
<td>human leukocyte antigen</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>mAbs</td>
<td>monoclonal antibodies</td>
</tr>
<tr>
<td>MBP</td>
<td>mannose-binding protein</td>
</tr>
<tr>
<td>NCR</td>
<td>noncoding region</td>
</tr>
<tr>
<td>NS</td>
<td>nonstructural protein</td>
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<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
<td>-----------------------------</td>
</tr>
<tr>
<td>ORF</td>
<td>single open reading frame</td>
</tr>
<tr>
<td>prM</td>
<td>precursor membrane</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>TS</td>
<td>type-specific</td>
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CHAPTER 1

INTRODUCTION
EPIDEMIOLOGY

Dengue is an acute viral disease transmitted between humans and mosquitoes, including the principal *Aedes* mosquito vector, *A. aegypti* and the secondary vector, *A. albopictus*. Dengue has been present for centuries. The first case of a dengue-like syndrome was recorded in 992 A.D. in China, and the first epidemic occurred in Philadelphia in 1780. Until World War II, four serotypes of dengue viruses (DENV1, 2, 3, 4) were suggested to be the viral etiology of dengue (1). During the World War II, the movement of troops between countries led to the spread of the viruses and their mosquito vectors to Southeast Asia and the West Pacific. Since then, in most Southeast Asian countries, dengue has remained hyperendemic (the presence of multiple serotypes of DENV cocirculating in one location) for all four serotypes (1). Prior to 1970, the campaign of elimination of *A. aegypti* effectively restricted the transmission of dengue in the Americas. However, the campaign was discontinued and the incidence of dengue in the Americas has increase since 1980 (1).

An estimated more than 3 billion people, half of the population in the world, are at the risk of DENV infection in tropical and subtropical regions (2). It was estimated that 390 million infections occur every year, including 96 million apparent infections and 500,000 cases of severe dengue, mostly among children, with the fatality rate more than 5 % in some areas (2-4). In the last 50 years, the incidence of dengue has increased 30-fold with a rapid geographic expansion across the world (2, 3). Prior to 1970, the severe form of disease, dengue hemorrhagic fever (DHF), has been reported in only five countries in
Southeast Asia. However, now DHF has been documented in more than 60 countries, and DENV is endemic in more than 100 countries, including Southeast Asia, South and Central America, and the Caribbean and South Pacific regions. The rapid spread of DENV in tropical and subtropical regions is due to the spread of mosquito vectors, increased urbanization, travel between countries, and ineffective vector control (1-3). Dengue epidemics have caused a significant economic and health burden. The global burden of dengue is now similar to the disease burden of other tropical diseases, including tuberculosis, in endemic countries in Asia and the Americas (2, 3).

**CLINICAL DISEASE**

Infection by DENV leads to a range of clinical manifestations, from asymptomatic infection to a self-limited febrile illness, dengue fever (DF), and severe form of disease, DHF and dengue shock syndrome (DSS). In general, 75-90% of infections are asymptomatic (1, 4). Most symptomatic manifestations present as classic DF. After an incubation period of 4 to 7 days, patients present with a sudden onset of fever accompanied by headache, pain behind the eyes, myalgia, arthralgia, rash, anorexia, abdominal pain, and nausea. Thrombocytopenia, leukopenia, and elevation in serum transaminases are also common in laboratory reports (2, 5). Most patients fully recover from DF. However, a small proportion of patients proceed to the severe forms of dengue disease, DHF/DSS, around the time of defervescence. DHF and DSS present as fever, thrombocytopenia, hemorrhage, and increased vascular permeability. The
hemorrhagic manifestations of DHF/DSS include capillary fragility, petechiae, eccymoses, purpura, or bleeding from the mucosa and gastrointestinal tract. Increased vascular permeability leads to the loss of plasma fluids into the interstitial spaces and results in pleural effusion and ascites. Hypovolemic shock occurs followed by sufficient leakage of plasma fluids into the interstitial spaces. The course of shock is short but life threatening. Patients usually recover within 24 hours under the appropriate medical care (2, 5).

**GENOME AND VIRUS STRUCTURE**

DENV genome consists of a single-stranded, positive-sense RNA of approximately 11 kb in length; there is a type I cap (m7G5'ppp5'A) at the 5' end but no polyadenylate tail at the 3' end. The genome encodes a single open reading frame (ORF) flanked by the 5’ and 3’ noncoding regions (NCR) of approximately 100 and 450 nucleotides, respectively. The terminal NCRs play key roles in viral translation and RNA replication. Translation of the single ORF produces a large polyprotein that is co- or post-translational cleaved by viral and host proteases into ten proteins. The N terminal one-fourth of genome encodes three structural proteins, capsid (C), precursor membrane (prM) which is further cleaved into pr and membrane (M) protein during maturation, and envelope (E). The remaining three-fourths of genome encodes seven non-structural proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (6) (Fig. 1A).

The virion of DENV, approximately 50 nm in diameter (Fig. 1B), is composed of an outer protein shell with 180 copies of the E protein and M protein,
and a host-derived lipid bilayer, which surrounds the inner nucleocapid consisting of the C protein and genomic RNA (6). Studies of cryo-electron microscopy and image reconstruction have shown that mature DENV particles have a relatively smooth outer surface with 90 E protein homodimers arranged into 30 rafts, each containing three parallel dimers, in the “herringbone” pattern (7, 8). On the other hand, immature DENV particles display 60 trimeric spikes, each consisting of prM-E heterodimers, on the surface (8, 9).

REPLICATION CYCLE

The major cell types infected by DENV in humans have been suggested to be monocytes, macrophages, and dendritic cells (10-12). The replication of DENV occurs in the cytoplasm of target cells. During virus entry, E proteins of DENV bind to receptors on the surface of host cells, leading to the internalization of DENV through clathrin-mediated endocytosis (13, 14). Although several cell surface molecules have been demonstrated the capability of mediating virus attachment, a specific receptor for DENV entry has not been identified. Inside the endosomes, the low pH environment triggers the structural rearrangement of E protein that results in the exposure of fusion peptides, driving the fusion between viral membrane and endosomal membrane (15, 16). Following the uncoating of nucleocapsid, viral RNA genome is released into the cytoplasm, and directly translated into a polyprotein that is cleaved by cellular and viral protease into three structural proteins (C, prM, and E) and seven non-structural proteins (NS1, NS2A/B, NS3, NS4A/B, and NS5) (6). Non-structural proteins are involved in
RNA replication that occurs in the cytoplasmic replication complexes associated with perinuclear membranes (17). Immature viral particles are assembled, bud into the lumen of endoplasmic reticulum (ER), and transported through the secretory pathway to the Golgi apparatus. Under the low pH environment in the trans-Golgi, E protein undergoes conformational rearrangement that leads to the exposure of a furin cleavage site on the prM protein, which is processed to pr and M protein by furin, resulting in the formation of mature viral particles (7, 8, 18, 19). Mature viral particles are released at the cell surface by exocytosis (Fig. 2).

PATHOGENESIS

The understanding of the mechanisms that lead to severe dengue are hindered due to the lack of an animal model accurately presenting the capillary permeability syndrome seen in patients after DENV infection. Epidemiologic studies have identified several risk factors that are associated with severe dengue, including age, ethnicity, possible chronic diseases, host factors and viral factors. It was reported that vascular leakage was more severe in young children than in adults, resulting in a greater risk of DSS (20). Host factors including female sex, several human leukocyte antigen (HLA) alleles, and polymorphisms in tumor necrosis factor (TNF) gene and the promoter of DC-SIGN receptor gene (21) have been associated with the risk of severe dengue disease. Moreover, the virulence of the infecting viral strain; the sequence of DENV infections, such as infection with DENV1 followed DENV2 or 3; or infection with DENV3 followed by
DENV2; secondary DENV infection, and the longer time interval between primary and secondary DENV infections have been suggested to correlate with more severe dengue diseases (22-24).

The immune response to DENV infection not only mediates protection against disease but also appear to be a major factor in the pathogenesis of DHF and DSS. A phenomenon called antibody-dependent enhancement (ADE) has been proposed to describe the role of the antibody response in the pathogenesis. Previous studies have shown that DHF/DSS was associated with a greater viremia in the blood by 10 to 100 folds compared with DF (25), and secondary infection with a heterotypic DENV correlated with DHF/DSS in humans (23, 25-27). In addition, DHF/DSS was observed in infants during primary DENV infection at the age of 6 to 12 months when the maternal antibodies (Abs) to DENV declined below the neutralizing level (28, 29). It was hypothesized that ADE accounts for increased viral replication of Fc\(\gamma\) receptor bearing cells, such as dendritic cells and monocytes or macrophages, leading to severe dengue (DHF/DSS) during secondary DENV infection (30-32). ADE is believed to occur when preexisting cross-reactive, non-neutralizing or weakly neutralizing Abs generated from primary DENV infection, or acquired passively at birth, bind to the heterotypic (different serotypes) DENV during secondary infection and the uptake of antibody-virus immune complex into Fc\(\gamma\) receptor bearing cells is greatly increased, that leads to enhanced viral replication. ADE has been demonstrated experimentally in vivo. In AG129 interferon (IFN) receptor-deficient mice, passive transfer of anti-E or anti-prM Abs enhanced dengue-like disease and increased
viral replication (33, 34). Passive transfer of specific dilution of polyclonal sera or monoclonal anti-E Abs also enhanced DENV replication in monkeys (35, 36).

Other mechanisms by which anti-DENV Abs contribute to severe disease have been proposed. First, complement activation by virus-antibody complexes was observed in DHF cases and the level of complement activation products C3a and C5a was associated with the severity of dengue disease (37). Second, it was reported that Abs against the E protein cross-react with human plasminogen, and Abs against the NS1 protein can bind to human coagulation proteins or proteins expressed by endothelial cells, leading to activation of coagulation pathway and alteration of endothelial cell function (38, 39).

Increased T cell activation and cytokine production have been reported in DHF patients during secondary DENV infection (40-42). It is possible that the T cell response also contribute to the pathogenesis of dengue. It has been proposed that during a secondary heterotypic DENV infection, massive activation of memory T cells following increased viral replication mediated by ADE results in the production of high level of pro-inflammatory cytokines such as tumor necrosis factor α (TNFα) and interleukin 2 (IL-2) that target endothelial cells and induce systemic vascular leakage (42, 43). Several studies have observed high concentrations of cytokines including IFN-γ, TNFα, and IL-2, 6, 1, 8, and 10 in patients with severe dengue disease (44, 45). In addition, it was reported that memory T cells had a lower affinity for the infecting DENV serotype and a higher affinity for the previous DENV serotype during secondary infection, so called
original antigenic sin (46). This response causes less efficient elimination of the infected cells and leads to severe disease.

**E PROTEIN**

The E protein, present on the surface of DENV virion, is the major target of neutralizing and enhancing Abs. The amino sequence similarity of the E proteins is 60-70% between serotypes (8) and 90-96% within each serotype (47-50). The ectodomain of E protein consists of three distinct domains: domains I, II and III (DI, DII and DIII) (16). DI is the central domain and forms into an eight-stranded β-barrel. DII, an elongated domain, is involved in dimerization and contains a highly conserved fusion loop (FL) that promotes fusion of viral and endosomal membranes during entry. DIII, an immunoglobulin-like domain, is believed to participate in receptor binding (6, 15, 51).

Anti-E Abs that recognize members of different serocomplexes in the genus Flavivirus, such as Japanese-encephalitis virus serocomplex and tick-borne encephalitis virus serocomplex, members within a serocomplex and a single member are called flavivirus group-reactive (GR), complex-reactive (CR) and type-specific (TS), respectively (52). Previous studies of mouse anti-E monoclonal antibodies (mAbs) against DENV have reported that GR mAbs primarily recognize the highly conserved residues in the FL of DII and are non-neutralizing or weakly neutralizing, while CR and TS mAbs recognized DIII or interdomain residues and has potent neutralizing activities (53-61). Recently, several studies have identified the epitopes recognized by human anti-E mAbs.
The epitopes of human GR anti-E mAbs were mapped to FL and adjacent to the bc loop (62-64); several TS and potent neutralizing mAbs were reported to recognize DIII (65, 66), interdomain region (67, 68) or DI/II hinge region (69) (Fig. 1C). However, the role of different categories of Abs in protection or pathogenesis after DENV infection has not been identified.

PROTECTIVE IMMUNE RESPONSES

The adaptive immune responses against DENV infection are believed to play the key role in protection against reinfection. Both humoral and cellular immune responses contribute to protection against DENV infection. However, there have been fewer studies of T cell-mediated protection than antibody-mediated protection. It is known that neutralizing Abs are the main mediators of protection against DENV infection; thus the main goal of DENV vaccine development is to induce protective level of neutralizing Abs.

Immune responses against both homotypic (the same serotype) and heterotypic (different serotypes) DENV are observed after primary or secondary DENV infections. The functional neutralization assay demonstrates that after primary DENV infection, individuals develop only monotypic neutralizing Abs against the infecting serotype (70-74), while following secondary DENV infection, individuals develop neutralizing Abs not only against the serotypes to which they have been exposed previously, but also against the serotypes to which they have not yet been exposed (32). It has been suggested that the induction of robust neutralizing antibody response after primary DENV infection provide life-long...
protection against reinfection with the same DENV serotype (70-74). Previous studies have reported that a repeat infection with the same DENV serotype that results in illness in endemic areas is rare (75), and illness caused by DENV in infants less than six months old is infrequent in endemic areas, which indicates that passively transferred maternal Abs can protect the infant (28, 76, 77). Volunteers challenged with DENV wild-type virus after primary infection also indicated that homotypic immunity was present for at least 18 months (longest interval evaluated) (70). Secondary DENV infection with a different DENV serotype is usually associated with severe disease. However, in third or fourth DENV infections, the frequency of severe disease is decreased as demonstrated by the very low numbers of hospital admission in humans (78) as well as the low rate of viremia in monkeys after a third DENV infection (79-82).

The principal targets of the antibody response after DENV infection in humans are E, prM, and NS1. Abs against NS3 and NS5 have been also detected. However, the responses were weak (83). Anti-E Abs are the predominant antibody response, and recognize multiple domains of the E protein. Anti-prM and anti-NS1 Abs are highly cross-reactive. Passive transfer of mAbs against the E protein has been showed to protect against DENV infection in mice and the protection is associated with high neutralizing activity (84). Protection against DENV challenge by passive transfer of mAbs against prM, NS1, and NS3 in mice has been observed (85-87). However, the mechanisms involved in the protection are not fully understood, and antibody-dependent cellular cytotoxicity and complement-mediated cell cytotoxicity may play a role (88, 89).
THE CURRENT DEVELOPMENT OF DENGUE VACCINES

A safe and effective vaccine is urgently needed to control dengue since the increasing emergence and expansion of areas where dengue is endemic. Progress towards developing a dengue vaccine has been slow during the course of 50 years, despite great efforts in research. Other than being free from significant reactogeneicinity after immunization, universal coverage in endemic areas, especially young children, and affordable cost, an ideal dengue vaccine must provide life-long protection, and induce protective immunity against all 4 DENV serotypes simultaneously to avoid the risk of waning immunity associated with the potential enhancement of disease (5).

There have been two types of approaches used for the development of dengue vaccines, including live, attenuated virus vaccines and nonliving (nonreplicating) dengue vaccines (5, 43, 90, 91). Several candidate vaccines are currently in clinical development.

Tetravalent live attenuated vaccines are the main approach for dengue vaccines currently and have advanced the furthest in clinical trials. Generally two strategies are used for live attenuated virus vaccines, including generation of an antigenic chimeric virus with a well-characterized attenuated flavivirus backbone or a mutated dengue virus that replicates inefficiently in humans but is still capable of inducing an immune response to structural and non-structural proteins. The recent ChimeriVax (CYD) dengue vaccine candidates under development by Sanofi Pasteur are chimeric virus vaccines in which the licensed live attenuated yellow fever virus (YFV) vaccine is modified by replacing the prM and E genes by
those derived from DENV (92) to generate four monovalent vaccine candidates. The results from phase 1 and 2 studies have suggested that tetravalent CYD vaccines are safe and immunogenic, and induce neutralizing antibody responses following immunization of 3 doses of vaccines at 4-6 month intervals (93), and currently the vaccines have entered phase 3 clinical trials in Thailand. The National Institute of Allergy and Infectious Disease (NIAID) Laboratory of Infectious Disease has developed live attenuated dengue vaccines by introducing a deletion into the 3' NCR region of full-length DENV cDNA clones (94), and successfully attenuated DENV1 and DENV4. However, this approach did not suitably attenuate DENV2 and DENV3, and an alternative chimeric strategy was used for DENV2 and DENV3 vaccines by substituting the prM and E genes from DENV2 and 3 for the corresponding genes of the attenuated DENV4 backbone. Phase 1 clinical trials have characterized and evaluated monovalent candidate vaccines for each of the 4 serotypes (95-98), and demonstrated tetravalent formulations of these vaccines are safe and can induce a trivalent or tetravalent neutralizing antibody response in 75-90% of vaccines (99). Additional tetravalent chimeric vaccines developed by Inviragen, which used attenuated DENV2 strain (16681-PDK53) as backbone (100), is currently undergoing phase 1 clinical trials in the U.S. and Columbia.

To avoid the issue of viral interference observed in the testing of live attenuated dengue vaccines, several strategies have been used to develop nonliving dengue vaccines, including inactivated whole virus vaccines (101-103), virus-like particle (VLP) vaccines (104), DNA vaccines (105) or virus-vector
vaccines (106, 107) that express DENV prM and E genes, and subunit vaccines containing purified E proteins (108) or DIII of E protein (109). A phase 1 clinical trial has been conducted by Merck/Hawaii Biotech to evaluate the monovalent DENV1 recombinant E subunit vaccine. Their data suggest that the vaccine is safe and immunogenic (108). Another alternative approaches such as epitope-modified vaccines that present potently neutralizing epitopes and eliminate or mask the poorly neutralizing epitopes, have been proposed and are undergoing preclinical development (110-112). Nonliving vaccines are promising if they can induce sustained antibody response in humans and can be produced economically.

Although the most advanced CYD tetravalent live attenuated dengue vaccine developed by Sanofi Pasteur has progressed to large-scale phase 3 clinical trials, the results from phase 2b trial appeared to be disappointing. Surprisingly, the vaccine showed an overall efficacy of 30.2% (efficacy of 80-90% against DENV3 and DENV4; efficacy of 60% against DENV1). However, the vaccine had no protection against DENV2 (113). While several dengue vaccine candidates have progressed to phase 2 or 3 clinical trials, the challenges associated with the limited understanding of the immune correlates of protection in humans and the factors contributing to disease severity during natural DENV infection still remain. More investigations are needed to better understand the immune responses and define the DENV epitopes that are involved in the polyclonal antibody responses in humans after DENV infection.
HYPOTHESIS AND SPECIFIC AIMS

The long-term goal of this study is to facilitate the development of a safe and effective DENV vaccine, such as epitope-modified vaccines that present neutralizing epitopes and remove non-neutralizing and enhancing epitopes. The objective of the proposed research is to elucidate the categories and epitopes of human anti-E Abs that are responsible for neutralization during natural DENV infection. The central hypothesis of this study is that after primary DENV infection TS anti-E Abs are responsible for neutralization against the infecting serotype, whereas after secondary DENV infection, GR anti-E Abs, the major component of cross-reactive Abs, correlate with neutralization against the non-exposed serotypes.

The objective is achieved by pursuing the following two specific aims:

Specific Aim 1: Define the role of TS anti-E Abs in the neutralizing activities against the infecting serotype and their epitopes in polyclonal sera after primary DENV infection

Specific Aim 2: Investigate GR anti-E Abs, the major component of cross-reactive Abs after primary and secondary DENV infections
A. To compare the neutralizing potency, binding avidity, and neutralization mechanisms of human GR anti-E mAbs derived from individuals following primary and secondary DENV infections

B. To determine the concentrations of GR anti-E Abs in polyclonal human sera after secondary DENV infection and the relationship to neutralizing activities against non-exposed serotypes
FIG 1. DENV genomic structure, mature virion, and E domains. (A) The genomic structure of DENV. RNA genome is translated to a polyprotein that is processed by viral and cellular proteases into three structural proteins and seven nonstructural proteins. (B) E proteins are present as homodimers on the surface of the mature virus particles. (C) The structural domains identified within the E protein: domain I in red, domain II in yellow, and domain III in blue. The highly conserved fusion loop (FL) and absolutely conserved bc loop residues in domain II are showed in green and yellow colors respectively (adapted from Murphy BR and Whitehead SS, 2011 (5)).
FIG 2. The replication cycle of DENV. (A) Virions bind to receptors on the cell surface and enter cells through endocytosis. (B) E protein undergoes conformational changes in the low pH of the endosome, and mediates fusion of viral and endosomal membranes. RNA genome is released into the cytoplasm. (C) Viral RNA genome is translated into a polyprotein that is processed by viral and cellular proteases. (D) Viral non-structural proteins replicate the genome RNA. (E) Virus assembles at the ER membrane to form immature virus particles. (F) Immature virions are transported through the secretory pathway. In the low pH of the trans-Golgi, prM is cleaved by furin, and drives maturation of the virus. (G) Mature virus is released into the cytoplasm (adapted from Perera et. al, 2008 (114)).
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CHAPTER 2

STUDY OF ANTIBODIES AGAINST THE ENVELOPE PROTEIN OF DENGUE VIRUS IN POLYCLONAL HUMAN SERA AFTER PRIMARY INFECTION
ABSTRACT

The four serotypes of dengue viruses (DENV) are the leading cause of arboviral diseases worldwide. Currently, there is no licensed DENV vaccine available. After primary DENV infection, individuals develop life-long protection against the infecting serotype, but only transient protection against the non-exposed serotypes. The type-specific neutralizing antibodies (Abs) against the exposed serotype are believed to account for the life-long protection, however, the epitopes of Abs that contribute to such neutralizing activities in polyclonal human sera remain largely unclear. In this study, we investigated the Abs against the envelope (E) protein of DENV in 10 individuals who experienced primary DENV1 infection during the 2001 outbreak in Hawaii. Depletion of cross-reactive Abs did not affect the DENV1 neutralizing activities in sera, whereas depletion of DENV1 type-specific Abs greatly reduced the DENV1 neutralization titers by 67% to 83%. The type-specific Abs constituted 16% to 41% of anti-E Abs in these sera, but accounted for the type-specific neutralizing activity after primary DENV infection. In addition, the requirement of virions rather than solubilized E protein alone to deplete the neutralizing activities in sera suggests the importance of neutralizing epitopes present on virions. After depletion of anti-DIII Abs, the reduction in neutralizing activity was 20% to 34% in four out of ten sera, suggesting the contribution of anti-DIII Abs to type-specific neutralizing activities. This observation resonates with previous reports of several potent neutralizing human monoclonal Abs targeting DIII. Our findings add to a better and in-depth
understanding of antibody responses after primary DENV infection and have implications for future dengue vaccine development.

INTRODUCTION

The four serotypes of dengue virus (DENV) are the leading cause of arboviral diseases in humans in tropical and subtropical regions (1, 2) with an estimated 390 million infections every year (3). The clinical manifestation after DENV infection ranges from asymptomatic, to an acute self-limiting febrile illness known as dengue fever (DF), or a severe, life threatening disease known as dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) (2, 4). Currently, there is no licensed vaccine available.

The E protein of DENV, present on the surface of virion, is the major target of neutralizing and enhancing antibodies (Abs) (5). The ectodomain of E protein consists of three distinct domains: domains I, II and III (DI, DII and DIII) (6). DI is the central domain. DII is involved in dimerization and contains a highly conserved fusion loop (FL) that promotes fusion of viral and endosomal membranes during entry. DIII is believed to participate in receptor binding (7-9).

Anti-E Abs that recognize members of different serocomplexes in the genus Flavivirus, such as Japanese-encephalitis virus serocomplex and tick-borne encephalitis virus serocomplex, members within a serocomplex and a single member are called flavivirus group-reactive (GR), complex-reactive (CR) and type-specific (TS), respectively (10). Previous studies of mouse anti-E monoclonal antibodies (mAbs) against DENV have reported that GR mAbs
primarily recognize the highly conserved residues in the FL of DII and are non-neutralizing or weakly neutralizing, while CR and TS mAbs recognize DIII or interdomain residues (11-19) and have potent neutralizing activities. Recently, several studies have identified the epitopes recognized by human anti-E mAbs. The epitopes of human GR anti-E mAbs were mapped to FL and adjacent bc loop (20-22); several TS and potent neutralizing mAbs were reported to recognize DIII (23, 24), interdomain region (25, 26), or DI/II hinge region (27).

After primary DENV infection, individuals develop TS neutralizing Abs against the infecting serotype, which can be detected 60 years after infection (28), suggesting these neutralizing Abs provide life-long protection against reinfection with the same serotype (29-31). However, the epitopes recognized by the TS neutralizing Abs in polyclonal human sera remain largely unknown. Previously, it was reported that the TS neutralizing activities in human sera after primary DENV infection cannot be depleted by recombinant DIII-MBP fusion protein (32). In addition, DENV containing DIII mutations can be neutralized by human sera after primary infection (33). These findings suggest that anti-DIII Abs do not contribute to such neutralizing activities.

Since recombinant DIII-MBP fusion protein may not preserve the correct conformation of DIII in DENV virions and DIII mutant viruses covering only two regions were tested previously (32, 33), the possibility that anti-DIII Abs contribute to neutralizing activities against the infecting serotype after primary DENV infection cannot be completely ruled out. Moreover, several potent neutralizing and TS anti-DIII mAbs have been produced from humans (23-25). We
hypothesize that TS anti-DIII Abs may contribute to such neutralizing activities. In this study, we investigated anti-E Abs in polyclonal human sera from 10 patients with primary DENV1 infection during the 2001 DENV1 outbreak in Hawaii, including their binding specificity, neutralizing activities before and after depletion with non-exposed serotype antigens, DENV1 virions and DENV1 DIII.

MATERIALS AND METHODS

Human sera

With the approval of the Institutional Review Board of the University of Hawaii at Manoa (CHS#17568), sera were collected in 2006 and 2008 from patients with primary DENV1 infection during the 2001 outbreak in Hawaii (34). The primary infection status was confirmed by monotypic neutralization pattern using plaque reduction neutralization test (PRNT) as described previously (28).

Generation of virions and cell lysates

Four serotypes of DENV, DENV1 (Hawaii strain), DENV2 (NGC strain), DENV3 (H87 strain), and DENV4 (H241 strain) were grown in Vero cells. Culture supernatants containing viruses were harvested 4-7 days after infection and centrifuged at low speed to remove cell debris. Clarified supernatants were subjected to 20% sucrose cushion ultracentrifugation using a Beckman SW 28 rotor at 25,000 rpm at 4°C for 5 h. Virus pellets were suspended in 1X PBS overnight, inactivated by UV, and used as antigens in depletion experiments and ELISA. Infected cells were washed with 1X PBS and lysed with 1% NP40 lysis
buffer (100 mM Tris, pH7.5, 150 mM NaCl, 20 mM EDTA, 1% NP40), followed by centrifugation at 2,000 g at 4°C for 30 min to obtain cell lysates that were used as the antigens in depletion experiments, dot blot and western blot analysis (19).

**Depletion experiment**

For depletion of cross-reactive antibodies, 900 μl of polyclonal human serum at dilution of 1:10 or 1:20 was incubated with a nitrocellulose membrane square (2.5 by 2.5 cm) pre-coated with cell lysates derived from DENV3-, DENV4- or mock-infected Vero cells at RT for 2 h, followed by further incubation with DENV3 or DENV4 virions or mock in solution at 37°C for 1 h. The virus-antibody complexes were pelleted down by ultracentrifugation in a Beckman TLA 120.1 rotor at 65,000 rpm at 4°C for 1h. The supernatants were collected as first depleted sera for further analyses. For deletion of TS antibodies, the first depleted sera were incubated with nitrocellulose membranes coated with DENV1 cell lysates at RT for 2 h, followed by incubation with DENV1 virions at 37°C for 1 h. The virus-antibody complexes were pelleted as described above. The supernatants were collected as second depleted sera for further analyses.

**Virion-ELISA, dot blot assay and Western blot analysis**

In order to detect anti-DENV antibodies in human sera before and after depletion, virion-ELISA, dot blot, and Western blot analysis were performed as described previously (19). For virion-ELISA, the plates were coated at 4°C overnight with four serotypes of DENV and West Nile virus (WNV) virus-like
particles (VLPs), which were derived from the ultracentrifugation of culture supernatants of 293 T cells transfected with a plasmid expressing prM and E proteins of WNV. The plates were washed with 1X PBS containing 0.2% Tween-20 prior to blocking (Thermo Scientific blocking buffer) for 1 h. Human sera at dilution of 1:500 were added and incubated at 37°C for 2 h, followed by the addition of HRP-conjugated anti-human IgG at 37°C for 1 h, and TMB substrate and stop solution at final step (19). The absorbance at wavelength of 450 nm (OD450) with reference wavelength of 650 nm was read. For dot blot and Western blot analysis, cell lysates derived from DENV-infected Vero cells were dotted to nitrocellulose membrane or subjected to 12% polyacrylamide gel electrophoresis (PAGE) under non-reducing condition. After blocking, nitrocellulose membranes were incubated with non-depleted and depleted sera at the dilution of 1:500, followed by the addition of secondary antibody, HRP-conjugated anti-human IgG (19). A mouse GR mAb (FL0231), which recognizes the E protein of 4 DENV serotypes and WNV similarly well (19), was used to adjust and confirm comparable amounts of loading antigens in virion-ELISA, dot blot, and Western blot analysis.

Focus reduction neutralization test (FRNT)

The neutralization titers of human sera before and after depletion were determined by FRNT<sub>50</sub>, the sera dilution at which 50 % reduction in foci was observed (21). Briefly, two-fold serial dilutions of serum were incubated with 50 focus forming units (FFU) of DENV1 (Hawaii strain) at 37°C for 1 h, and added to
Vero cell monolayers, grown in a 96-well plate. After virus adsorption at 37°C for 1.5 h, cells were covered with methylcellulose medium, and incubated at 37°C. Four days later, methylcellulose overlay was removed, and cells were washed with 1X PBS, fixed, and stained with mAb 4G2. Foci were counted by the CTL Immunospot Analyzer. The FRNT<sub>50</sub> titers were determined by a sigmoidal dose-response curve with variable slopes (GraphPad Prism 5.0).

**Determination of the proportion of TS Abs (% TS Abs) in human serum**

ELISA plates were coated with DENV1 virons, washed, and blocked. Four-fold serial dilutions of mock- or DENV3/4-depleted sera were added and incubated at 37°C for 2 h, followed by incubation with HRP-conjugated anti-human IgG at 37°C for 1 h, and TMB substrate and stop solution at final step. Cell lysates and pellets derived from mock-infected Vero cells were used as control in the depletion protocol. The absorbance at wavelength of 450 nm (OD<sub>450</sub>) with reference wavelength of 650 nm was read. The endpoint titers were calculated as the reciprocal of the serum dilution that yielded a signal greater than 3 standard deviation of the signal from a pool of 5 normal human sera. The % DENV1-TS Abs was calculated by the endpoint titer of DENV3/4-depleted serum divided by that of mock-depleted serum.

**Detection of anti-DIII Abs by DIII-ELISA**

ELISA plates were coated with 100 ng of DENV1 recombinant DIII per well (WestPac strain) (kindly provided by Dr. Michael S. Diamond) (35) at 4°C
overnight, and washed prior to blocking at RT for 1 h. Mock-depleted and DIII-depleted sera were added and incubated at 37°C for 2 h followed by the incubation of secondary Ab HRP-conjugated anti-human IgG at 37°C for 1 h. TMB substrate and 2N H2SO4 stop solution were added, and the absorbance at wavelength of 450 nm (OD450) with reference wavelength of 650 nm was read. A mouse mAb (DV1E99) that recognizes DIII of E protein (kindly provided by Dr. Michael S. Diamond) (18) was used to adjust the amount of loading antigen.

DIII depletion

Cyanogen bromide (CNBr)-activated beads (Sigma) were washed with ddH2O followed by coupling buffer (0.1 M NaHCO3 in 0.5 M NaCl, pH 8.5), and then 120 μg of DENV1 recombinant DIII (WestPac strain) diluted in coupling buffer was incubated with 5 mg of hydrated CNBr-activated beads at 4°C overnight. Control beads were incubated with coupling buffer. DIII-conjugated or control beads was washed with coupling buffer prior to blocking (0.2 M glycine) at RT for 2 h. In order to remove the blocking buffer completely, beads were washed with coupling buffer (pH 8.5) followed by 0.1 M acetate buffer (pH 4) for four times. After wash with 1X PBS twice, DIII-conjugated beads were mixed with 200 μl (1:10 or 1:20) of first depleted serum and incubated at 37°C for 2 h. DIII-depleted sera were collected after centrifugation of beads at low speed.
RESULTS

Reduction in DENV1 TS neutralizing activity in human sera by depletion with DENV1 but not with other serotypes’ antigens

We studied DENV-immune sera collected between 2006 and 2008 from 10 individuals, who experienced primary infection during the DENV1 outbreak in Hawaii in 2001. Neutralization pattern of these sera determined by PRNT$_{90}$ was consistent with previous history of primary DENV1 infection (Table 1). To investigate the category of anti-E Abs in polyclonal sera that contribute to the neutralizing activities, we employed an approach of stepwise depletion to separate different categories of anti-E Abs. Human sera were incubated with DENV3 or DENV4 (non-exposed serotypes) antigens followed by DENV1 (exposed serotype) antigens including cell lysates of DENV1-infected cells and virions. Undepleted and mock-depleted sera bound to E proteins of four DENV serotypes and West Nile virus (WNV) in dot blot assay, Western blot analysis and virion-ELISA (Figs. 1A and 1C); this is in agreement with previous reports of predominant cross-reactive anti-E Abs in human sera after primary DENV infection (32, 36, 37). Depletion of sera with DENV4 antigens greatly reduced the binding activities to DENV2, DENV4, WNV and DENV3 to a lesser extent, and only the DENV1 TS binding activity remained, suggesting that our protocol successfully remove cross-reactive anti-E Abs (GR plus CR) from polyclonal sera (Figs. 1A and 1C). Depletion of cross-reactive anti-E Abs did not reduce the neutralization titers of sera (Figs. 1B and 1D). In contrast, further depletion with DENV1 antigens greatly reduced the DENV1 TS binding activity and DENV1 TS
neutralization titers by 67 to 83% in the 5 serum samples tested (Figs. 1B, 1D and 1E). The results suggest that TS anti-Abs in polyclonal sera but not cross-reactive Abs, though present in abundance, were responsible for the neutralizing activities against the infecting serotype after primary DENV infection.

To test whether the complex structure of E proteins on DENV1 virion is required for depleting the TS neutralizing activities in human sera, we performed a modified depletion experiment, in which incubation with DENV3 or DENV4 antigens was followed by DENV1 antigen containing DENV1-infected cell lysates containing only solubilized E proteins presented as prM-E heterodimers or E homodimers (38-41)(Figs. 2A and 2C). Interestingly, depletion with DENV1-infected cell lysate antigen reduced the E-binding activity to DENV1 (Figs. 2A and 2C) but not the neutralization activity (Figs. 2B, 2D and 2E), suggesting that the structure of E proteins on virions, which is not preserved in solubilized E proteins in cell lysates, is required to deplete the TS neutralizing activity. These findings were in agreement with previous reports that several TS potently neutralizing human mAbs bind to virions only but not recombinant E protein, suggesting the importance of the E structures on virions for the recognition by potent TS neutralizing mAbs (25, 26).

The relationship between the titers of TS binding Abs and TS neutralizing Abs titers

We next determined the proportion of TS Abs (% TS Abs) in polyclonal human sera by DENV1 virion ELISA using serially four-fold dilutions of
mock-depleted and DENV3 and DENV4-depleted sera. Based on the binding curves, the endpoint titers were determined using the OD values of a pool of 5 normal sera plus 3 standard deviations as cut-off in a nonlinear regression analysis (Graphpad) (Fig. 3A). The endpoint titers of mock-depleted sera represented the titers of total anti-E Abs including GR, CR, and TS Abs, whereas that of DENV3/DENV4-depleted sera represented the titers of DENV1 TS Abs since cross-reactive Abs (GR plus CR) were removed. The % DENV1 TS Abs in sera was calculated by the endpoint titer of mock-depleted sera divided by that of DENV3/DENV4-depleted sera (Fig. 3A). The % DENV1 TS Abs in the 10 serum samples tested ranged from 15.6% to 40.8% (Fig. 3B). We also used another method to calculate the % DENV1 TS Abs. The OD value of DENV3/DENV4-depleted serum at the dilution of 1:500 was interpolated to the non-linear regression binding curve generated from the mock-depleted serum to determine the relevant serum dilution and calculate the % DENV1 TS Abs (Figs. 3A and 3B). The % DENV1 TS Abs calculated by the endpoint titer method correlated with that by the interpolation method (spearman r = 0.72, p = 0.002) (Fig. 3C). In addition, the titers of TS binding Abs had a trend of positive correlation with the TS neutralizing Abs titers, though it was not significant probably due to small sample size (Fig. 3D). This finding suggests that TS Abs were responsible for the TS neutralizing activities in polyclonal human sera after primary DENV infection.

The contribution of TS anti-DIII Abs to TS neutralizing activities in sera
To examine whether anti-DIII Abs contribute to TS neutralizing activities in human sera after primary DENV1 infection, we performed a depletion experiment using recombinant DIII of DENV1 Western Pacific strain, the same genotype that the individuals were exposed to during the 2001 outbreak in Hawaii. After depletion with antigens of DENV3 and DENV4 (non-exposed DENV serotypes) including cell lysates plus virions to remove cross-reactive Abs, the sera were further depleted with recombinant DIII of DENV1. Compared with mock-depletion, depletion of human sera with recombinant DIII led to great decrease of DIII-binding activities to the level below the cut-off value of the DIII-ELISA, suggesting nearly complete removal of anti-DIII Abs in these sera (Figs. 4A, 4B, and 4C, left panels). However, depletion of anti-DIII Abs did not affect the TS neutralization titers in 6 out of 10 serum samples; whereas, the TS neutralizing titers were reduced by 16.7% to 39.8% in other 4 cases (Figs. 4A, 4B, 4C right panels and 4D). Additionally, compared with mock depletion, depletion with DENV3/DENV4 antigens to remove cross-reactive Abs resulted in reduction in the DIII-binding activities; this suggests that some anti-DIII Abs are cross-reactive (Figs. 4A, 4B, and 4C, left panels).

**DISCUSSION**

In this study, we employed a protocol of depletion with antigens of non-exposed DENV serotype followed by antigens of exposed DENV serotype to demonstrate that TS anti-E Abs, rather than cross-reactive anti-E Abs, contribute to the TS neutralizing activities in polyclonal human sera after primary DENV
infection. The conformation on virions is required to deplete such neutralizing Abs. Moreover, we determined the % TS Abs in human sera by the endpoint titers of anti-DENV1 Abs post-depletion; the titers of TS binding Abs had a trend of positive correlation with the TS neutralizing Abs titers in polyclonal human sera. Our depletion experiment of anti-DIII Abs by recombinant DIII of DENV1 genotype IV, the same genotype that the patients were infected with, showed that the TS neutralizing activities were not affected in six out of ten serum samples tested but reduced in other four serum samples.

Previously, we and others reported that cross-reactive anti-E Abs constituted a large proportion of anti-DENV Abs in polyclonal human sera after primary DENV infection and only a minor proportion was TS or anti-DIII Abs (32, 36, 37). Recently, de Alwis et al. reported that TS Abs rather than cross-reactive Abs are responsible for the TS neutralizing activity in human sera after primary DENV infection (25). The TS neutralizing activities can be depleted by virions but not solubilized E proteins of the infecting serotype; which was consistent with the binding property of two potent neutralizing human mAbs that bound to virions but not to the recombinant soluble E proteins. Teoh et al. performed cryo-EM study and reported that a TS potent neutralizing human mAb 14c10, isolated from a patient with primary DENV1 infection, recognized a discontinuous epitope across the adjacent E protein dimers (26). Our findings that TS Abs accounted for the TS neutralizing activity are in agreement with the report using similar depletion experiment by de Alwis et al (25). Moreover, the requirement of virions to deplete the TS neutralizing Abs from human sera was consistent with the importance of a
complex quaternary epitope recognized by potent neutralizing TS human mAbs (25, 26). Interestingly and importantly, we developed a method to measure the % TS Abs and titers of TS Abs in human sera and found that the titers of TS binding Abs had a trend of positive correlation with the TS neutralizing Abs titers in polyclonal human sera. Future studies involving a larger sample size are needed to verify this observation and demonstrate that the % TS Abs and titers of TS Abs in human sera could be potential and useful parameters of correlate of protection after primary DENV infection.

Previous studies of mouse mAbs have suggested that DIII of E protein is the main target of many potent neutralizing Abs against DENV (12-19). Studies of human mAbs isolated from individuals who recovered from primary DENV infection revealed that several potent neutralizing TS human mAbs recognized DIII or DI/DII hinge region of E protein, and some cross-reactive human mAbs also recognized DIII (23-25, 42, 43). Our observations that removal of cross-reactive anti-E Abs reduced the DIII-binding activity of human sera suggest the presence of cross-reactive anti-DIII Abs in DENV immune sera. In addition, it was reported recently that TS neutralizing Abs in the sera from 77% of vaccinees receiving monovalent DENV1 live-attenuated vaccine recognize an epitope (E126, E157) close to the DI/DII hinge region, as the mutations of these two residues but not the DI/DII hinge residues reduced the sensitivity to neutralization by the vaccinee sera (44). Although DIII may not be the immunodominant region recognized by TS neutralizing Abs in polyclonal human sera, the possibility that TS anti-DIII Abs may still contribute to protection cannot be completely excluded.
Our findings that depletion of TS anti-DIII Abs reduced the TS neutralizing activity in sera by 20% to 34% in four out of ten cases with primary DENV1 infection suggest the contribution of anti-DIII Abs to TS neutralizing activity in human sera during natural DENV infection.

ACKNOWLEDGMENTS

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FIG 1. Depletion with DENV1 but not other serotypes’ antigens reduce DENV1 TS neutralizing activity in sera of patients with primary DENV1 infection. (A,C) Dot blot (DB) assay, Western blot (WB) analysis and virion-ELISA were performed for undepleted, mock (Mo)-depleted, D4-depleted and D4-D1-depleted sera from patients ID 24 (A) and ID9 (C) as described in Methods. Mouse mAb FL0231, which recognizes the FL, was used to control the comparable amounts of virion antigens. (B,D) FRNT\textsubscript{50} to DENV1 for the above sera from patients ID24 (B) and ID9 (D). (E) Summary of % reduction in FRNT\textsubscript{50} titers to DENV1 in 5 patients with primary DENV1 infection.
FIG 2. Depletion with DENV1 antigen derived from DENV1-infected cell lysates does not reduce DENV1 TS neutralizing activity in sera of patients with primary DENV1 infection. (A,C) Dot blot (DB) assay was performed for undepleted, mock (Mo)-depleted, D3 (or D4)-depleted and D3(or D4)-D1-depleted sera from patients ID8 (A) and ID29 (C) as described in Methods. (B,D) FRNT$_{50}$ to DENV1 for the above sera from patients ID8 (B) and ID29 (D). (E) Summary of % reduction in FRNT$_{50}$ titers to DENV1 in 3 patients with primary DENV1 infection.
E

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FIG 3. Determination of the proportion of DENV1 TS Abs and the relationship to the titers of DENV1 TS neutralizing Abs in sera of patients with primary DENV1 infection. (A) Serially two-fold dilutions of mock (Mo)-depleted and DENV3 and DENV4 (D3/D4)-depleted sera were subjected to DENV1 virion-ELISA, and the endpoint titers were determined as described in Methods. The % DENV1 TS Abs was calculated by the formula, endpoint titer of Mo-depleted sera divided by that of D3/D4-depleted sera, or interpolation of the OD values of D3/D4-depleted sera to the non-linear regression curves based on the Mo-depleted serum as described in Methods. (B) Summary of the % DENV1 TS Abs in sera of 10 patients calculated by two methods. (C) The relationship between the titers of DENV1 TS binding Abs and DENV1 TS neutralizing Abs titers.
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B

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C

Spearman $r = 0.72$
$p = 0.002$

D

Spearman $r = 0.35$
$p = 0.33$
FIG 4. The contribution of TS anti-DIII Abs to DENV1 TS neutralizing activities in sera of patients with primary DENV1 infection. (A,B,C)

DENV1-derived DIII-ELISA was performed for mock (Mo)-depleted, DENV3 and DENV4 (D3/D4)-depleted, D3/D4-Mo-depleted and D3/D4-DIII-depleted sera from patients ID5, ID9 and ID35 (C) (left panels of A, B and C, respectively). The neutralization curves (middle panels) and the FRNT$_{50}$ titers (right panels) to DENV1 were shown. (D) Summary of % reduction in FRNT$_{50}$ titers to DENV1 after depletion with D3/D4-DIII in sera from 10 patients with primary DENV1 infection.
**A**

- OD 450 nm
- % Neutralization
- FRNT to D1

**B**

- OD 450 nm
- % Neutralization
- FRNT to D1

**C**

- OD 450 nm
- % Neutralization
- FRNT to D1

**D**

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<tr>
<td>#10</td>
<td>0 ± 6</td>
</tr>
<tr>
<td>#29</td>
<td>0 ± 6</td>
</tr>
<tr>
<td>#35</td>
<td>0 ± 10</td>
</tr>
</tbody>
</table>

**Explanation:**

- The table shows the reduction in FRNT<sub>50</sub> titers to D1 for different patient IDs after depletion with D3/D4+Mo or D3/D4+DIII.
- Values are presented as mean ± standard deviation.
TABLE 1. Basic information and neutralization titers of patients with primary DENV1 infection

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Time of sampling</th>
<th>PRNT$_{90}$ titers$^c$</th>
<th>D1</th>
<th>D2</th>
<th>D3</th>
<th>D4</th>
</tr>
</thead>
<tbody>
<tr>
<td># 9</td>
<td>5 to 7 years</td>
<td>160</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td># 24</td>
<td>5 to 7 years</td>
<td>160</td>
<td>20</td>
<td>&lt; 20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td># 26</td>
<td>5 to 7 years</td>
<td>320</td>
<td>20</td>
<td>&lt; 20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td># 27</td>
<td>5 to 7 years</td>
<td>320</td>
<td>&lt; 20</td>
<td>20</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td># 5</td>
<td>5 to 7 years</td>
<td>320</td>
<td>&lt; 20</td>
<td>20</td>
<td>&lt; 20</td>
<td></td>
</tr>
<tr>
<td># 23</td>
<td>5 to 7 years</td>
<td>320</td>
<td>&lt; 20</td>
<td>&lt; 20</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td># 8</td>
<td>5 to 7 years</td>
<td>160</td>
<td>&lt; 20</td>
<td>&lt; 20</td>
<td>&lt; 20</td>
<td></td>
</tr>
<tr>
<td># 10</td>
<td>5 to 7 years</td>
<td>160</td>
<td>&lt; 20</td>
<td>&lt; 20</td>
<td>&lt; 20</td>
<td></td>
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<tr>
<td># 29</td>
<td>5 to 7 years</td>
<td>320</td>
<td>40</td>
<td>&lt; 20</td>
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<tr>
<td># 35</td>
<td>5 to 7 years</td>
<td>320</td>
<td>40</td>
<td>&lt; 20</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$Ten adult patients with primary DENV1 infection during the 2001 outbreak in Hawaii were included in this study (Imrie et al., 2007).
$^b$Serum and plasma samples were collected between 2006 and 2008 and analyzed.
$^c$The neutralizing titers were determined by 90% inhibition on PRNT (PRNT$_{90}$) on Vero cells (28). D1: DENV1; D2: DENV2; D3: DENV3; D4: DENV4.
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CHAPTER 3

HIGH AVIDITY AND POTENT NEUTRALIZING CROSS-REACTIVE HUMAN MONOCLONAL ANTIBODIES DERIVED FROM SECONDARY DENGUE VIRUS INFECTION

Journal of Virology 2013, 87 (23): 12562-12575
ABSTRACT

The envelope (E) protein of dengue virus (DENV) is the major target of neutralizing antibodies (Abs) and vaccine development. Previous studies of human dengue-immune sera reported that a significant proportion of anti-E Abs, known as group-reactive (GR) Abs, were cross-reactive to all four DENV serotypes and to one or more other flaviviruses. Based on studies of mouse anti-E monoclonal antibodies (mAbs), GR mAbs were non-neutralizing or weakly neutralizing compared with type-specific mAbs; a GR response was thus not regarded as important for vaccine strategy. We investigated the binding avidities, and neutralization potencies of 32 human GR anti-E mAbs. The neutralization potencies and binding avidities of GR mAbs derived from secondary DENV infection were stronger than those derived from primary infection. GR mAbs derived from primary DENV infection primarily blocked attachment, whereas those derived from secondary infection blocked DENV post-attachment. Analysis of the repertoire of anti-E mAbs derived from patients with primary DENV infection revealed that the majority were GR, low-avidity, and weakly neutralizing mAbs, whereas those from secondary infection were primarily GR, high-avidity, and potently neutralizing mAbs. Our findings suggest that the weakly neutralizing GR anti-E Abs generated from primary DENV infection become potently neutralizing mAbs against the four serotypes after secondary infection. The observation that the dengue immune status of the host affects the quality of the cross-reactive Abs generated has implications for new strategies for DENV vaccination.
INTRODUCTION

The four serotypes of dengue virus (DENV) are the leading cause of arboviral diseases in humans in tropical and subtropical regions (1, 2). More than 2.5 billion people in over 100 countries were estimated to be at risk of infection and 50 to 100 million DENV infections occurred every year worldwide (1, 2). After DENV infection, most individuals are asymptomatic or present with a self-limited illness, known as dengue fever, some may develop severe and potentially life-threatening diseases, known as dengue hemorrhagic fever/dengue shock syndrome. Despite considerable efforts to develop vaccines, several being tested in clinical trials, there is no licensed dengue vaccine currently available (1, 3).

DENV belongs to the genus *Flavivirus* in the family *Flaviviridae*. It is a positive-sense, single-stranded RNA virus with a genome of approximately 10.6 kilobases in length. The genome contains a single open reading frame encoding a polyprotein, which is cleaved by cellular and viral proteases into three structural proteins, the capsid, precursor membrane (prM) and envelope (E), and seven non-structural proteins (4). After binding to its cellular receptor, DENV enters the cell through receptor-mediated endocytosis (5, 6). Under the low pH environment of endosome, E protein undergoes a series of conformational changes, leading to the fusion of viral membrane to endosomal membrane and virus entry (6, 7).

The E protein is present as 90 “head-to-tail” homodimers on the surface of mature virions (4, 6, 8). E protein participates in virus entry and is the major target of neutralizing as well as enhancing antibodies (Abs) (9). In the presence of cross-reactive non-neutralizing or suboptimal concentration of neutralizing Abs,
DENV replicates to higher titers in human Fcγ receptor-bearing cells in vitro, a phenomenon known as antibody-dependent enhancement (9, 10). The ectodomain of E protein contains three domains (7). Domain I is located in the center. Domain II contains a fusion loop (FL) at the tip and is involved in membrane fusion and dimerization of E protein. Domain III is believed to participate in receptor binding (4, 6, 11).

In the genus Flavivirus, there are several serocomplexes, including the DENV serocomplex, Japanese-encephalitis virus serocomplex, and tick-borne encephalitis virus serocomplex (4). Anti-E Abs that recognize members from different serocomplexes, members within a serocomplex and a single member are called flavivirus group-reactive (GR), complex-reactive (CR) and type-specific (TS), respectively (12). Previous studies of mouse anti-E monoclonal antibodies (mAbs) against DENV have reported different epitopes and neutralizing potency for different categories of mAbs. GR mAbs primarily recognized the highly conserved residues in the FL of domain II, and were non-neutralizing or weakly neutralizing compared to several potent neutralizing TS (13-16). Therefore, GR response was regarded as useless for vaccine strategy.

In this study, we investigated the binding avidity and neutralization potency of 32 human GR anti-E mAbs. We found the neutralization potency and binding avidity of GR mAbs derived from patients with secondary DENV infection were stronger than those derived from primary infection. Analysis of the repertoire of anti-E mAbs derived from four individuals revealed predominant low-avidity and weakly neutralizing GR mAbs from patients with primary DENV
infection and predominant high-avidity and potent neutralizing GR mAbs from patients with secondary infection. These findings suggest that the weakly neutralizing GR anti-E Abs generated from primary DENV infection become potent neutralizing after secondary infection and have implications for alternative vaccine strategies against DENV.

MATERIALS AND METHODS

Human mAbs and binding specificity

With the approval of the Institutional Review Board of the University of Hawaii at Manoa (approval CHS#17568), 28 human anti-E mAbs derived from four patients with primary DENV infection (by each of the four DENV serotypes) and 23 anti-E mAbs derived from four patients with secondary infection were included in this study (Table 1). The mAbs from patients DVG, DVD, DVC, and DVB were generated by a B-cell immortalization protocol as described previously (17, 18) and were provided by the Pediatric Dengue Vaccine Initiative. The mAbs from patients 749, 751, 753, and 750 were generated at the Imperial College of London by a previously described reverse transcription-PCR (RT-PCR) and cloning protocol for antibody-secreting cells derived from peripheral blood mononuclear cells (PBMCs) (19), in compliance with the guidelines of the Institutional Ethical Committee, and were shipped to the University of Hawaii at Manoa in August 2010 for analysis. Binding specificity was determined by dot blot assay (20) or Western blot analysis (21, 22).
**Binding avidity by virion-capture ELISA**

Virion-capture ELISA was performed in the same way as VLP-capture ELISA (13, 22), except that virions derived from ultracentrifugation of culture supernatants of infected Vero cells were used as antigen. The dissociation constant ($K_d$) was determined by the program GraphPad Prism 5.0. DENV1 virion-capture ELISA was performed for all GR mAbs, and DENV4 virion-capture ELISA was performed for the repertoire of mAbs (4 TS and 6 GR MAbs) from a DENV4 case. A linear relationship was found between the $K_d$ of 6 GR MAbs determined by DENV4 virion-capture ELISA and that determined by DENV1 virion-capture ELISA (correlation coefficient $[r] = 0.83; \ P = 0.029$ by the two-tailed Spearman correlation test) (data not shown), supporting the use of the $K_d$ determined by DENV1 virion-capture ELISA to compare binding avidities among GR mAbs.

**Focus reduction neutralization test (FRNT) and mechanism of neutralization**

The neutralization potency of each mAb was determined by the concentration that gave 50% inhibition in a focus reduction neutralization test ($\text{FRNT}_{50}$ value) (13). Four-fold serial dilutions of each mAb were incubated with 50 focus-forming units (FFU) of each serotype and added to Vero cell monolayers grown in a 96-well plate. The four serotypes included DENV1 (Hawaii strain), DENV2 (NGC strain), DENV3 (CH53489), and DENV4 (H241 strain) grown in Vero cells. Foci were counted by use of a CTL immunospot
analyzer 3 to 4 days later. The FRNT\textsubscript{50} titers were determined by a nonlinear regression analysis (GraphPad Prism 5.0). For the mechanism of neutralization, mAbs were added at pre- and postattachment steps to study the mechanism of neutralization as described previously (13, 23). The relative amount of infection was determined as follows: \% infection = number of foci in the presence of mAb/number of foci in the absence of mAb.

**Competitive virion-capture ELISA**

A previously described mouse GR MAb (E53) recognizing FL (24) was prepared at 0.002 \( \mu \text{g/ml} \) (corresponding to its mean \( K_d \) value for four DENV serotypes), mixed with or without each human GR MAb (at 0.25 \( \mu \text{g/ml} \), corresponding to the mean \( K_d \) for GR MAbs to DENV1), and subjected to virion-capture ELISA using DENV1 (Hawaii strain), DENV2 (NGC strain), DENV3 (H87 strain), or DENV4 (H241 strain), derived from ultracentrifugation of culture supernatants of infected Vero cells, with anti-mouse IgG as a secondary Ab (25). Inhibition was determined as follows: \% inhibition = (1 − OD\textsubscript{450} of E53 alone/OD\textsubscript{450} of E53 plus human GR MAb) × 100.

**Relative binding activity after low pH treatment**

A flat-bottom 96-well plate was coated with DENV1 virions at 4°C overnight, followed by blocking with 1% BSA in 1× PBS for 1 h and the addition of anti-E mAb at pH 7.4 and 37°C for 2 h; the concentration near maximum binding was used because of the requirement of higher occupancy to reach
neutralization by FL mAbs (15). After washing twice with 1× PBS and once with the indicated buffer at pH 7.4 (blocking buffer) or pH 5.0 (150 mM NaCl and 50 mM MES [morpholineethanesulfonic acid]), followed by incubation with pH 7.4 or pH 5.0 buffer at room temperature for 30 min (23) and washing with 1× PBS twice, ELISA was completed by the regular capture ELISA protocol (13, 22). The relative binding activity after low-pH treatment was determined as follows: % binding activity = OD450 at pH 5.0/OD450 at pH 7.4.

**Statistical analysis**

The two-tailed Mann-Whitney test was used to determine the difference in Kd, FRNT50 and relative binding activity after low pH treatment between two groups, and two-tailed Spearman correlation test was used to determine the relationship between Kd and FRNT50 by GraphPad Prism 5.0.

**RESULTS**

**Stronger neutralization potency of GR anti-E mAbs derived from secondary DENV infection than those derived from primary infection**

We examined the neutralization potencies of these 32 mAbs by FRNT (13). As shown in Table 3, the concentrations that gave 50% inhibition in FRNT (FRNT50) for most mAbs derived from patients with primary DENV infection were ≥1 μg/ml against four DENV serotypes, whereas the FRNT50 titers of mAbs derived from patients with secondary infection were generally lower than 1 μg/ml. The FRNT50 concentrations of mAbs derived from secondary DENV infection
were significantly lower than those derived from primary infection ($P < 0.0001$, $P = 0.0005$, $P = 0.004$, and $P = 0.01$ for DENV1, DENV2, DENV3, and DENV4, respectively, by the two-tailed Mann-Whitney test), indicating that the GR anti-E mAbs derived from secondary DENV infection were more potent neutralizing mAbs than those derived from primary infection.

**Higher binding avidity of GR anti-E mAbs derived from secondary DENV infection than those derived from primary infection**

Since the 32 GR mAbs recognized the E proteins of four DENV serotypes similarly well (data not shown), we examined the binding avidities of these mAbs by using a DENV1 virion-capture ELISA and also determined the dissociation constant ($K_d$). As shown in Fig. 1A, the $K_d$ values of mAbs derived from patients with secondary DENV infection were significantly lower than those of mAbs derived from patients with primary infection ($P = 0.0001$ by the two-tailed Mann-Whitney test), indicating that GR anti-E mAbs derived from secondary DENV infection had higher binding avidities than those derived from primary infection.

To exclude the possibility that the different epitopes (either FL residues only or both FL and bc loop residues) may account for the differences in binding avidity and neutralizing potency, we first compared the binding curves of three mAbs recognizing the same epitope involving four FL residues (W101, G106, L107, and F108) (Table 2). As shown in Fig. 1B, the $K_d$ values of the two mAbs (749B2 and 751C4) derived from secondary DENV infection were lower than that of the mAb (DVB64.31) derived from primary infection. Consistent with this, the
mean FRNT<sub>50</sub> concentration of these two mAbs was lower than that of the mAb derived from primary DENV infection (Fig. 1B and Table 3). We next compared the binding curves of seven mAbs recognizing similar epitopes involving three FL residues (three residues among W101, F108, G106, and L107) (Table 2). As shown in Fig. 1C, the \( K_d \) values of the five mAbs derived from secondary DENV infection were lower than those of the two mAbs derived from primary infection; this is consistent with the stronger neutralization potencies of these five mAbs (Fig. 1C and Table 3). A similar trend was observed for six mAbs recognizing epitopes involving both FL and bc loop residues, in that the binding avidities and neutralization potencies of three mAbs derived from secondary DENV infection were greater than those of three mAbs derived from primary infection (Fig. 1D and Table 3). Taken together, these findings suggest that the higher binding avidities of GR mAbs derived from secondary DENV infection, recognizing either FL residues or both FL and bc loop residues, may account for their stronger neutralization potencies.

To assess the binding avidities of these GR mAbs for each of the four DENV serotypes by a different assay, we employed a previously described competitive virion-capture ELISA in which a known concentration of each human GR mAb competed with a mouse GR mAb (E53) recognizing FL (24, 25). We found that the % inhibition in DENV1, DENV2, DENV3, and DENV4 competitive virion-capture ELISAs by GR mAbs derived from secondary infection was significantly greater than that by GR mAbs derived from primary infection \( (P = 0.019, P < 0.0001, P = 0.0001, \) and \( P = 0.0002 \) for DENV1, DENV2, DENV3, and
DENV4 competitive virion-capture ELISAs, respectively, by the two-tailed Mann-Whitney test) (Fig. 2A to D), suggesting higher binding avidities to all four serotypes for GR mAbs derived from secondary infection. Notably, a linear relationship was found between the $K_d$ values and FRNT$_{50}$ titers against the four serotypes ($P < 0.0001$, $P < 0.0001$, $P = 0.0001$, and $P < 0.0001$ for DENV1, DENV2, DENV3, and DENV4, respectively, by the two-tailed Spearman correlation test) (Fig. 3). These findings suggest that the higher binding avidities of GR mAbs derived from secondary DENV infection may account for the stronger neutralization potencies against four DENV serotypes than those of mAbs derived from primary infection (Table 3).

**Mechanism of neutralization**

To investigate if the mechanisms of neutralization by GR mAbs derived from primary and secondary DENV infections are different, we performed a previously described pre- and post-attachment neutralization assay for 3 GR mAbs recognizing the same FL epitope (residues W101, G106, L107, and F108) (Table 2) (11, 23). As shown in Fig. 4B, the GR mAb derived from primary DENV infection, DVB 64.31, had greater inhibition in the pre-attachment assay than in the post-attachment assay, suggesting that it neutralizes DENV mainly by blocking attachment. The pattern was similar to that of a control mouse mAb, 3H5, as reported previously (Fig. 4A) (11). In contrast, two GR mAbs (751C4 and 749B2) derived from secondary DENV infection showed similar inhibition both pre-attachment and post-attachment, suggesting that at least part of their
neutralization was at the post-attachment step (Fig. 4C and D) (23). This
observation was further verified by another 3 GR mAbs (DVD19.4 and DVD19.13,
derived from primary DENV infection, and 749B12, derived from secondary
infection) recognizing a similar FL epitope (three residues among W101, F108,
G106, and L107) (Fig. 4E to G).

The possibility that GR mAbs derived from secondary DENV infection,
which have high binding avidities at neutral pH, continue to bind virions tightly in
the low-pH environment in the endosome compared to those derived from
primary infection, and thus can block the post-attachment step, was further
examined by a virion-capture ELISA performed at neutral pH followed by low-pH
incubation, which mimics the steps of entry from attachment at neutral pH to
fusion at low pH. As shown in Fig. 4H, the relative binding activities after low-pH
treatment of 6 GR mAbs derived from secondary DENV infection were
significantly higher than those of 6 GR mAbs derived from primary infection ($P =
0.009$ by the two-tailed Mann-Whitney test), suggesting that the stronger binding
activity of GR mAbs derived from secondary infection after low-pH treatment may
account for their ability to neutralize DENV at the post-attachment step in the
endosome.

**Repertoire of anti-E mAbs derived from patients with primary and
secondary DENV infections**

Previous studies of polyclonal human sera after DENV infection revealed
that a significant proportion of anti-E Abs were GR Abs, whereas a minor
proportion were TS Abs (13, 21, 22, 26). To better understand the anti-E Ab responses after primary DENV infection, we examined the repertoire of anti-E mAbs (including TS, CR, and GR mAbs) derived from two patients with primary infection, namely, DVG and DVD (Table 1). The five mAbs derived from DVG, a patient with primary DENV1 infection, included four GR mAbs and one DENV1-TS mAb. As shown in Fig. 5A, the TS mAb had a higher binding avidity to DENV1 than three of the four GR mAbs. Consistent with this, the TS mAb had stronger neutralization potency against DENV1 than the four GR mAbs. The 12 mAbs derived from DVD, a patient with primary DENV4 infection, included 6 GR mAbs, 2 CR mAbs, and 4 DENV4-TS mAbs. Because of the presence of DENV4-TS mAbs in this case, DENV4 virion-capture ELISA and FRNT against DENV4 were performed and compared. The mean $K_d$ value of the TS mAbs was lower than that of the GR mAbs (Fig. 5B). Similarly, the mean FRNT$_{50}$ concentration of TS mAbs was lower than that of the GR mAbs.

For comparison, we examined the repertoire of anti-E mAbs derived from two patients with secondary DENV infection, namely, patients 750 and 753 (Table 1). For the three mAbs derived from patient 750, a patient with secondary DENV1 infection, the binding avidities and neutralization potencies of two GR mAbs were higher than those of the CR mAb (Fig. 5C). For the six mAbs derived from patient 753, also a patient with secondary DENV1 infection, the mean $K_d$ and FRNT$_{50}$ concentration of five GR mAbs were lower than those of the CR mAb (Fig. 5D).
DISCUSSION

In this study, we investigated the neutralization potencies, and binding avidities of GR human anti-E mAbs derived from primary and secondary DENV infections. Based on previous studies of mouse anti-E mAbs against DENV, most GR mAbs were weakly neutralizing and thus not regarded as important responses for dengue vaccine strategies (14-16). However, when we compared GR human anti-E mAbs derived from different origins, we found that GR mAbs derived from patients with secondary DENV infection had stronger neutralization potencies and higher binding avidities than those of mAbs derived from patients with primary infection, suggesting that the weakly neutralizing GR anti-E Abs generated from primary DENV infection become potently neutralizing Abs after secondary DENV infection. This was further supported by analysis of the repertoire of anti-E mAbs derived from two patients with primary DENV infection and two patients with secondary DENV infection. To our knowledge, this is the first report showing that the dengue immune status of the host (primary versus secondary DENV infection) may affect the quality of cross-reactive Abs generated. These findings not only add to our understanding of human anti-E Abs after natural DENV infection but also have implications for alternative strategies for dengue vaccine development.

Previously, Beltramello et al. characterized the neutralizing and enhancing activities of 51 human anti-E mAbs derived from five dengue cases (18). Of the 27 mAbs derived from two patients with secondary DENV infection, 22 recognized domain I/II, cross-reacted to four DENV serotypes, and were
presumably GR mAbs. Of the 24 mAbs derived from three patients with primary DENV infection, most were TS or CR mAbs, and only 3 were presumably GR mAbs, based on their binding to domain I/II and cross-reactivity. Due to the small number of GR mAbs derived from primary DENV infection in the data set, a statistical analysis to compare the neutralization potencies of GR mAbs derived from primary and secondary DENV infections was not possible. As shown in Table 1, we studied all available 12 and 20 GR mAbs derived from primary and secondary DENV infections, respectively, excluding the possibility of selection bias in this study. Recent studies reported that temperature can affect the structure and neutralization of DENV (27-29) and that DENV strains grown in different cell types have different N-linked glycans (30). In this study, different binding assays and an FRNT assay (except for pre- and post-attachment assays in the mechanistic study) were all performed at 37°C, and the viruses used were all derived from Vero cells grown at 37°C. Therefore, it is unlikely that the correlation between $K_d$ and FRNT$_{50}$ (Fig. 3) observed will be affected by variations such as the temperature and sources of virus in these assays.

We and others reported previously that a significant proportion of anti-E Abs in polyclonal human sera after DENV infection were GR Abs and primarily recognized the FL of domain II, whereas only a minor proportion were TS Abs and recognized domain III (13, 21, 22, 26). In agreement with this, our analysis of the repertoire of anti-E mAbs derived from two patients with primary DENV infection revealed that the majority were GR mAbs and a minor proportion were TS or CR mAbs. Moreover, the binding avidities of TS mAbs were generally
greater than those of GR mAbs in these patients (Fig. 5A and B). The epitopes recognized by GR anti-E mAbs involved either FL residues only or both FL and bc loop residues; these residues were highly conserved by different flaviviruses and absolutely conserved by the four DENV serotypes. It is conceivable that during secondary DENV infection, memory B cells recognizing these highly conserved FL and/or bc loop residues expand greatly and generate high-avidity GR Abs through affinity maturation (31). In agreement with this interpretation, studies of polyclonal human sera after DENV infection, which contained a significant proportion of GR anti-E Abs, revealed that the avidity of serum anti-DENV Abs from secondary infection was higher than that of Abs from primary infection (32, 33). In addition, recent studies reported that cross-reactive memory B cells or plasma cells, as well as serum avidity, increased greatly during acute secondary DENV infection (33-35).

Investigation of the mechanism of neutralization by pre- and post-attachment assays revealed that GR mAbs derived from primary DENV infection neutralize DENV mainly at the attachment step, whereas GR mAbs derived from secondary infection can also neutralize DENV at the post-attachment step, probably by blocking membrane fusion in the endosome. A previous study of mouse mAbs reported that domain III mAbs (such as 3H5) primarily block virus at the attachment step and domain I-II mAbs (such as GR mAb 4G2) also block attachment, though to a lesser extent (11). Another study demonstrated that a potent domain III mAb against WNV, E16, can block virus at the post-attachment steps (23, 24). Since 3H5 and E16 recognized similar epitope residues in the
lateral ridge of domain III, the observations that 3H5 blocks attachment and E16 can block post-attachment steps suggest that a subtle difference in epitope or other factors, such as avidity, may account for different mechanisms of neutralization. In this regard, our findings suggest that the stronger binding activity after low-pH treatment for GR mAbs derived from secondary DENV infection than for those derived from primary infection may contribute to the ability to neutralize DENV at the post-attachment step.

After primary DENV infection, individuals developed life-long protection against the infecting serotype, which has been shown to correlate with the appearance of TS neutralizing Abs against that serotype (36, 37). After secondary DENV infection, individuals developed not only homotypic neutralizing Abs against the serotypes to which they had previously been exposed but also heterotypic neutralizing Abs against the serotypes to which they had not yet been exposed (9). Such heterotypic neutralizing Abs are believed to contribute to the very small numbers of hospitalization cases for humans after a third or fourth DENV infection (38) and to the low rate of viremia after a third DENV infection in monkeys (39, 40). Our finding that the weakly neutralizing GR anti-E Abs generated from primary DENV infection become potently neutralizing Abs after secondary infection suggests that such GR anti-E Abs, present as a predominant population, may contribute to the heterotypic neutralizing activities against the serotypes to which they have not yet been exposed. The possibility that some CR anti-E Abs generated after secondary DENV infection also contribute to such heterotypic neutralizing activities cannot be ruled out completely, though they
constituted a minor population and very few human CR mAbs are available for characterization thus far. In this regard, our analysis of 4 CR anti-E mAbs which recognized an epitope in the domain II central interface (residues Q211, D215, and/or P217; highly conserved in DENV serocomplex viruses) revealed that the binding avidities and neutralizing potencies of those derived from secondary DENV infection were stronger than those of mAbs derived from primary infection (data not shown). This provides more evidence supporting the hypothesis that the immune status of the host (primary versus secondary DENV infection) affects the quality of cross-reactive Abs generated.

There are several limitations of this study. First, the time of blood sampling to prepare mAbs in this study was either the acute phase (4 to 5 days), for GR mAbs derived from secondary DENV infection, or convalescent phase (1 month), for most (10 of 12 mAbs) of the GR mAbs derived from primary infection (Table 1). Whether the observations of higher binding avidities and neutralizing potencies of GR mAbs derived from secondary DENV infection than those of GR mAbs from primary infection hold true at later time points after DENV infection remains to be investigated. In this regard, it is worth noting that in the study of Beltramello et al., several potently neutralizing GR mAbs derived from secondary DENV infection were prepared at 7 to 17 months post-infection (18). Second, the DENV serotypes of primary infections were different. For the 12 GR mAbs derived from primary infection, 4 were from DENV1 infection, 6 from DENV4 infection, and 1 each from DENV2 and DENV3 infections (Table 1). For the 20 GR mAbs derived from secondary infections, the serotypes of the primary
Infections were not known. Whether the sequence of primary and secondary DENV infection affects the quality of GR mAbs generated remains to be investigated. Third, the donors were different, and the sample size was small. Fourth, the methodologies for generating these mAbs were different. Future studies with a larger sample size and using the same methodology to generate mAbs from the same subjects following their own sequential primary or secondary infection are needed to validate these observations.

Because of cocirculation of multiple DENV serotypes commonly seen in areas where dengue is endemic and the association of increased disease severity in persons during secondary DENV infection, the goal of current dengue vaccines is to attain balanced neutralizing Ab and T cell responses against all four DENV serotypes (1, 3, 41). Although several tetravalent live-attenuated dengue vaccines have moved to phase II or III clinical trials, a challenge of such an approach is the difficulty in simultaneously achieving balanced TS neutralizing Abs against all four DENV serotypes, due to dominant viremia by one or two serotypes resulting from interserotype interference (3, 41-43). Other approaches, such as epitope-modified vaccines presenting the potently neutralizing epitopes recognized by TS Abs and eliminating or masking the poorly neutralizing epitopes in the tetravalent format, have been proposed and reported recently (44-46). Our finding that secondary DENV infection greatly improves the quality of cross-reactive anti-E Abs generated suggests that an alternative approach of sequential heterotypic immunization (with nonreplicating dengue candidate vaccines) to mimic sequential natural infection might elicit potently neutralizing
Abs against all four DENV serotypes. It should be noted that a recent study reporting no efficacy for a tetravalent dengue vaccine against DENV2 in a phase IIb clinical trial raised concerns about the relevance of the \textit{in vitro} neutralization assay and the importance of T cell immunity in protection (43, 47). Although the \textit{in vitro} neutralization assay has been used as a surrogate of protective immunity in vaccine studies (reviewed in reference 3), several studies failed to show perfect correlation between neutralization and protection in mouse and nonhuman primate models (25, 48, 49). In contrast to previous studies showing T cell responses associated with severe dengue disease in humans (50, 51), a recent study of T cell responses against DENV suggested a protective role of CD8 T cells (52). These highlight the need to explore different functional assays of Abs and T cells to understand the immune correlates of protection and the application of better assays to vaccine studies.

**ACKNOWLEDGEMENTS**

We thank Gwong-Jen Chang at the Centers for Disease Control and Prevention at Fort Collins for kindly providing rabbit sera for capture ELISA and Saguna Verma at the University of Hawaii at Manoa for kindly providing lysates of WNV-infected cells. This work was supported by a cooperative research agreement from the International Vaccine Institute/Pediatric Dengue Vaccine Initiative and by grant P20GM103516 from the National Institute of General Medical Sciences, NIH.
FIG 1 Binding avidities and neutralization potencies of GR human anti-E mAbs. (A) Binding curves and $K_d$ values for 12 and 20 GR human anti-E mAbs derived from primary and secondary DENV infections, respectively, were determined by a DENV1 virion-capture ELISA. The FRNT$_{50}$ values against DENV1 are shown on the right. The two-tailed Mann-Whitney test was used to compare the two groups. (B to D) Binding curves, $K_d$ values, and FRNT$_{50}$ values against DENV1 for three GR human mAbs recognizing the same epitope involving four FL residues (W101, G106, L107, and F108) (B), seven GR human mAbs recognizing three FL residues (three residues among W101, F108, G106, and L107) as an epitope (C), and six GR human mAbs recognizing both FL residues and bc loop residues as an epitope (D). Data are means (with standard deviations for binding curves) for duplicates from one representative experiment of two. Blue closed symbols, mAbs derived from primary DENV infections; red open symbols, mAbs derived from secondary DENV infections.
FIG 2 Percent inhibition in competitive virion-capture ELISAs of four DENV serotypes by GR human anti-E mAbs. A mouse GR mAb (E53) recognizing FL (24) was prepared at 0.002 μg/ml (corresponding to its mean $K_d$ for the four DENV serotypes), mixed with or without each human GR MAb (at 0.25 μg/ml, corresponding to the mean $K_d$ for GR mAbs to DENV1), and subjected to competitive capture ELISA using DENV1 (A), DENV2 (B), DENV3 (C), or DENV4 (D) virions, with anti-mouse IgG as the secondary Ab (25). Inhibition was determined as follows: % inhibition = $\left( 1 - \frac{OD_{450} \text{ of E53 alone}}{OD_{450} \text{ of E53 plus human GR mAb}} \right) \times 100$. Data are means with standard deviations for two independent experiments (each in duplicate). The two-tailed Mann-Whitney test was used to compare the two groups. A linear relationship was found between the $K_d$ determined by DENV1 virion-capture ELISA and the % inhibition in DENV1 (E), DENV2 (F), DENV3 (G), and DENV4 (H) competitive virion-capture ELISAs. The two-tailed Spearman correlation test was performed using GraphPad Prism 5.0.
FIG 3 Relationship between $K_d$ and FRNT$_{50}$ values for GR mAbs against four DENV serotypes. The binding curves and $K_d$ values for 32 GR human anti-E mAbs were determined by a DENV1 virion-capture ELISA. Neutralization potencies were determined by the FRNT$_{50}$ values against DENV1 (A), DENV2 (B), DENV3 (C), and DENV4 (D). The two-tailed Spearman correlation test was performed using GraphPad Prism 5.0.
FIG 4 Mechanism of neutralization. (A to G) Pre- and post-attachment neutralization assays against DENV2 were performed as described previously (11, 23) for 3 GR mAbs recognizing the same FL epitope (residues W101, G106, L107, and F108), including DVB64.31 (B), derived from primary DENV infection, and 751C4 (C) and 749B2 (D), derived from secondary infection, and for 3 GR mAbs recognizing similar FL epitopes (three residues among W101, F108, G106, and L107), including DVD19.4 (E) and DVD19.13 (F), derived from primary infection, and 749B12 (G), derived from secondary infection. (A) A previously reported mouse mAb, 3H5, served as a control (11). The relative amount of infection was determined as follows: % infection = number of foci in the presence of mAb/number of foci in the absence of MAb. (H) DENV virion-capture ELISA was performed at pH 7.4, followed by incubation at pH 5.0 for 12 GR mAbs. The relative binding activity after low-pH treatment was determined as follows: % binding activity = OD_{450} at pH 5.0/OD_{450} at pH 7.4. The two-tailed Mann-Whitney test was used to compare the two groups. Data are means with standard deviations for three independent experiments (each in duplicate).
FIG 5 Binding avidities and neutralization potencies of anti-E mAbs from patients with primary and secondary DENV infections. (A to D) Binding curves, $K_d$ values, and FRNT$_{50}$ values for the repertoire of anti-E mAbs derived from two patients with primary DENV infection, DVG (A) and DVD (B), and two patients with secondary infection, 750 (C) and 753 (D). DENV1 virion-capture ELISA and FRNT against DENV1 were performed, except for DVD (a patient with primary DENV4 infection), for whom DENV4 virion-capture ELISA and FRNT against DENV4 were performed. Data are means (with standard deviations for binding curves) for duplicates from one representative experiment of two. Green open symbols, TS mAbs; blue closed symbols, GR mAbs; yellow closed symbols, CR mAbs; red open symbols, GR mAbs.
<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Type of infection</th>
<th>Serotype</th>
<th>Time of sampling</th>
<th>No. of anti-E MAbs (specificity)</th>
<th>No. of GR anti-E MAbs studied</th>
</tr>
</thead>
<tbody>
<tr>
<td>DVG</td>
<td>Primary</td>
<td>D1</td>
<td>1 mo</td>
<td>5 (4 GR, 0 CR, 1 TS)</td>
<td>4</td>
</tr>
<tr>
<td>DVD</td>
<td>Primary</td>
<td>D4</td>
<td>1 mo</td>
<td>12 (6 GR, 2 CR, 4 TS)</td>
<td>6</td>
</tr>
<tr>
<td>DVC&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Primary</td>
<td>D2</td>
<td>8 yr</td>
<td>10 (1 GR, 4 CR, 5 TS)</td>
<td>1</td>
</tr>
<tr>
<td>DVB&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Primary</td>
<td>D3</td>
<td>1 yr</td>
<td>1 (1 GR, 0 CR, 0 TS)</td>
<td>1</td>
</tr>
<tr>
<td>749</td>
<td>Secondary</td>
<td>D1</td>
<td>4 days</td>
<td>4 (4 GR, 0 CR, 0 TS)</td>
<td>4</td>
</tr>
<tr>
<td>751</td>
<td>Secondary</td>
<td>D1</td>
<td>4 days</td>
<td>10 (9 GR, 1 CR, 0 TS)</td>
<td>9</td>
</tr>
<tr>
<td>753</td>
<td>Secondary</td>
<td>D1</td>
<td>5 days</td>
<td>6 (5 GR, 1 CR, 0 TS)</td>
<td>5</td>
</tr>
<tr>
<td>750</td>
<td>Secondary</td>
<td>D1</td>
<td>5 days</td>
<td>3 (2 GR, 1 CR, 0 TS)</td>
<td>2</td>
</tr>
</tbody>
</table>

<sup>a</sup> Primary or secondary DENV infection and the infecting serotype of patients were determined as reported previously (17, 20). D, DENV.

<sup>b</sup> Time from the onset of illness to collection of blood to prepare mAbs

<sup>c</sup> Number of anti-E mAbs with known binding specificity and epitopes. GR, group-reactive; CR, complex-reactive; TS, type-specific.

<sup>d</sup> Ten mAbs were derived from patient DVC by use of a recombinant domain III selective subcloning method as described previously (17). Only one GR anti-E mAb was identified.
## TABLE 2 Epitopes of group-reactive anti-E human mAbs in this study

<table>
<thead>
<tr>
<th>mAb Derived From</th>
<th>Epitope(^a)</th>
<th>FL Residues Only</th>
<th>FL and BC Loop Residues</th>
</tr>
</thead>
<tbody>
<tr>
<td>DVD19.4</td>
<td>101, 106, 108</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DVD19.13</td>
<td>101, 106, 108</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DVD23.3</td>
<td>101, 106</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DVD23.4</td>
<td>101, 106</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DVB64.31</td>
<td>101, 106, 107, 108</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DVC23.13</td>
<td>101, 108</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DVG6.3</td>
<td>101, 108</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DVG7.5</td>
<td>101, 108</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DVG1.3</td>
<td>107</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DVG12.2</td>
<td>107, 108, 76</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DVD26.3</td>
<td>101, 107, 108, 78</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DVD26.11</td>
<td>101, 107, 108, 78</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DVD19.2</td>
<td>101, 106, 108</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DVD23.3</td>
<td>101, 106</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DVB64.31</td>
<td>101, 106, 107, 108</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DVC23.13</td>
<td>101, 108</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DVG6.3</td>
<td>101, 108</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DVG7.5</td>
<td>101, 108</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DVG1.3</td>
<td>107</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DVG12.2</td>
<td>107, 108, 76</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DVD26.3</td>
<td>101, 107, 108, 78</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DVD26.11</td>
<td>101, 107, 108, 78</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)Epitopes were identified by a previously described dot blot assay, VLP-capture ELISA, and structure-based analysis (22).
### TABLE 3 Neutralization potencies and binding avidities of group-reactive human anti-E mAbs in this study

<table>
<thead>
<tr>
<th>mAb</th>
<th>Patient ID, immune status(^a^)</th>
<th>D1</th>
<th>D2</th>
<th>D3</th>
<th>D4</th>
<th>(K_d) ((\mu)g/ml)(^b^)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DVG1.3</td>
<td>DVG, primary D1</td>
<td>1</td>
<td>0.25</td>
<td>&gt;1</td>
<td>1</td>
<td>0.021 ± 0.002</td>
</tr>
<tr>
<td>DVG6.3</td>
<td>DVG, primary D1</td>
<td>&gt;1</td>
<td>1</td>
<td>&gt;1</td>
<td>&gt;1</td>
<td>0.397 ± 0.078</td>
</tr>
<tr>
<td>DVG7.5</td>
<td>DVG, primary D1</td>
<td>1</td>
<td>1</td>
<td>&gt;1</td>
<td>&gt;1</td>
<td>0.777 ± 0.048</td>
</tr>
<tr>
<td>DVG12.2</td>
<td>DVG, primary D1</td>
<td>&gt;1</td>
<td>&gt;1</td>
<td>&gt;1</td>
<td>1</td>
<td>0.684 ± 0.052</td>
</tr>
<tr>
<td>DVD19.4</td>
<td>DVD, primary D4</td>
<td>&gt;1</td>
<td>1</td>
<td>&gt;1</td>
<td>0.25</td>
<td>0.199 ± 0.027</td>
</tr>
<tr>
<td>DVD19.13</td>
<td>DVD, primary D4</td>
<td>1</td>
<td>0.25</td>
<td>&gt;1</td>
<td>1</td>
<td>0.296 ± 0.037</td>
</tr>
<tr>
<td>DVD23.3</td>
<td>DVD, primary D4</td>
<td>1</td>
<td>1</td>
<td>&gt;1</td>
<td>0.25</td>
<td>0.207 ± 0.019</td>
</tr>
<tr>
<td>DVD23.4</td>
<td>DVD, primary D4</td>
<td>&gt;1</td>
<td>0.25</td>
<td>1</td>
<td>1</td>
<td>0.234 ± 0.024</td>
</tr>
<tr>
<td>DVD26.3</td>
<td>DVD, primary D4</td>
<td>&gt;1</td>
<td>1</td>
<td>&gt;1</td>
<td>1</td>
<td>0.509 ± 0.056</td>
</tr>
<tr>
<td>DVD26.11</td>
<td>DVD, primary D4</td>
<td>&gt;1</td>
<td>&gt;1</td>
<td>&gt;1</td>
<td>&gt;1</td>
<td>1.878 ± 0.178</td>
</tr>
<tr>
<td>DVC23.13</td>
<td>DVC, primary D2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0.065 ± 0.005</td>
</tr>
<tr>
<td>DVB64.31</td>
<td>DVB, primary D3</td>
<td>1</td>
<td>&gt;1</td>
<td>&gt;1</td>
<td>&gt;1</td>
<td>0.360 ± 0.030</td>
</tr>
<tr>
<td>749B2</td>
<td>749, secondary D1</td>
<td>1</td>
<td>0.25</td>
<td>1</td>
<td>0.25</td>
<td>0.052 ± 0.005</td>
</tr>
<tr>
<td>749B4</td>
<td>749, secondary D1</td>
<td>0.063</td>
<td>0.25</td>
<td>&gt;1</td>
<td>0.016</td>
<td>0.043 ± 0.003</td>
</tr>
<tr>
<td>749B6</td>
<td>749, secondary D1</td>
<td>1</td>
<td>1</td>
<td>&gt;1</td>
<td>&gt;1</td>
<td>0.032 ± 0.002</td>
</tr>
<tr>
<td>749B12</td>
<td>749, secondary D1</td>
<td>0.063</td>
<td>0.25</td>
<td>1</td>
<td>0.016</td>
<td>0.034 ± 0.003</td>
</tr>
<tr>
<td>751A1</td>
<td>751, secondary D1</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.047 ± 0.003</td>
</tr>
<tr>
<td>751A2</td>
<td>751, secondary D1</td>
<td>0.25</td>
<td>0.25</td>
<td>1</td>
<td>1</td>
<td>0.097 ± 0.005</td>
</tr>
<tr>
<td>751A12</td>
<td>751, secondary D1</td>
<td>1</td>
<td>1</td>
<td>&gt;1</td>
<td>1</td>
<td>0.052 ± 0.007</td>
</tr>
<tr>
<td>751B3</td>
<td>751, secondary D1</td>
<td>1</td>
<td>0.25</td>
<td>1</td>
<td>1</td>
<td>0.105 ± 0.009</td>
</tr>
<tr>
<td>751B6</td>
<td>751, secondary D1</td>
<td>0.063</td>
<td>0.063</td>
<td>0.83</td>
<td>1</td>
<td>0.025 ± 0.002</td>
</tr>
<tr>
<td>751B11</td>
<td>751, secondary D1</td>
<td>0.25</td>
<td>0.25</td>
<td>1</td>
<td>0.25</td>
<td>0.013 ± 0.001</td>
</tr>
<tr>
<td>751B12</td>
<td>751, secondary D1</td>
<td>1</td>
<td>0.25</td>
<td>1</td>
<td>0.25</td>
<td>0.022 ± 0.001</td>
</tr>
<tr>
<td>751C4</td>
<td>751, secondary D1</td>
<td>0.25</td>
<td>0.063</td>
<td>0.31</td>
<td>0.25</td>
<td>0.011 ± 0.001</td>
</tr>
<tr>
<td>751C10</td>
<td>751, secondary D1</td>
<td>0.063</td>
<td>0.063</td>
<td>1</td>
<td>0.063</td>
<td>0.019 ± 0.003</td>
</tr>
<tr>
<td>753A1</td>
<td>753, secondary D1</td>
<td>1</td>
<td>0.25</td>
<td>&gt;1</td>
<td>1</td>
<td>0.130 ± 0.010</td>
</tr>
<tr>
<td>753C1</td>
<td>753, secondary D1</td>
<td>0.25</td>
<td>0.063</td>
<td>0.25</td>
<td>0.25</td>
<td>0.015 ± 0.001</td>
</tr>
<tr>
<td>753C6</td>
<td>753, secondary D1</td>
<td>0.063</td>
<td>0.063</td>
<td>1</td>
<td>0.25</td>
<td>0.024 ± 0.002</td>
</tr>
<tr>
<td>753C8</td>
<td>753, secondary D1</td>
<td>1</td>
<td>0.25</td>
<td>1</td>
<td>1</td>
<td>0.063 ± 0.004</td>
</tr>
<tr>
<td>753C12</td>
<td>753, secondary D1</td>
<td>0.25</td>
<td>0.063</td>
<td>0.25</td>
<td>0.063</td>
<td>0.035 ± 0.003</td>
</tr>
<tr>
<td>750C9</td>
<td>750, secondary D1</td>
<td>0.25</td>
<td>1</td>
<td>&gt;1</td>
<td>1</td>
<td>0.296 ± 0.030</td>
</tr>
<tr>
<td>750D7</td>
<td>750, secondary D1</td>
<td>1</td>
<td>0.25</td>
<td>&gt;1</td>
<td>1</td>
<td>0.070 ± 0.006</td>
</tr>
</tbody>
</table>

\(^a^\)The immune status (primary or secondary DENV infection) and infecting serotype of patients were determined as reported previously (17, 20). D, DENV.

\(^b^\)The \(K_d\) was determined by DENV1 virion-capture ELISA as described in Materials and Methods.
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CHAPTER 4
ANALYSIS OF CROSS-REACTIVE ANTIBODIES RECOGNIZING THE FUSION LOOP OF ENVELOPE PROTEIN AND CORRELATION WITH NEUTRALIZING ANTIBODY TITERS IN NICARAGUAN DENGUE CASES

PLoS Neglected Tropical Diseases 2013, 7 (9): e2451
ABSTRACT

Dengue virus (DENV) is the leading cause of arboviral diseases in humans worldwide. The envelope (E) protein of DENV is the major target of neutralizing antibodies (Abs). Previous studies have shown that a significant proportion of anti-E Abs in human serum after DENV infection recognize the highly conserved fusion loop (FL) of E protein. The role of anti-FL Abs in protection against subsequent DENV infection versus pathogenesis remains unclear. A human anti-E monoclonal Ab was used as a standard in a virion-capture ELISA to measure the concentration of anti-E Abs, [anti-E Abs], in dengue-immune sera from Nicaraguan patients collected 3, 6, 12 and 18 months post-infection. The proportion of anti-FL Abs was determined by capture ELISA using virus-like particles containing mutations in FL, and the concentration of anti-FL Abs, and [anti-FL Abs] was calculated. Neutralization titers (NT_{50}) were determined using a previously described flow cytometry-based assay. Analysis of sequential samples from 10 dengue patients revealed [anti-E Abs] and [anti-FL Abs] were higher in secondary than in primary DENV infections. While [anti-FL Abs] did not correlate with NT_{50} against the current infecting serotype, it correlated with NT_{50} against the serotypes to which patients had likely not yet been exposed (“non-exposed” serotypes) in 14 secondary DENV3 and 15 secondary DENV2 cases. These findings demonstrate the kinetics of anti-FL Abs and provide evidence that anti-FL Abs play a protective role against “non-exposed” serotypes after secondary DENV infection.
INTRODUCTION

The four serotypes of dengue virus (DENV1–4) are the leading cause of arboviral diseases in humans in tropical and subtropical regions [1,2]. It has been estimated that more than 3 billion people in over 100 countries are at risk of infection and 50–100 million DENV infections occur annually worldwide [1,2]. The clinical presentation after DENV infection ranges from asymptomatic infection to a self-limited illness, dengue fever (DF), to severe and potentially life-threatening disease, dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) [1,2]. While considerable efforts have been made to develop interventions, currently there is no licensed vaccine or antiviral therapeutic against dengue available [30].

DENV belongs to the genus *Flavivirus* in the family *Flaviviridae*. It contains a positive-sense RNA genome of approximately 11 kilobases in length. Flanked by the 5’ and 3’ untranslated regions, the genome has a single open reading frame encoding a polyprotein precursor, which is cleaved by cellular and viral proteases into three structural proteins, the capsid, precursor membrane (prM) and envelope (E), and seven non-structural proteins [4]. The E protein forms 90 “head-to-tail” homodimers on the surface of mature virions [4–6]. The E protein participates in virus entry and is the major target of neutralizing antibodies (Abs) [3,4]. In the presence of non-neutralizing or suboptimal concentrations of neutralizing anti-E Abs, DENV replicates to higher titers in human Fcγ receptor bearing cells in vitro, a phenomenon known as antibody-dependent enhancement (ADE) [7–9]. The ectodomain of E protein contains three domains
Domain II contains an internal fusion loop (FL) that is involved in membrane fusion. Domain III is believed to participate in receptor binding [4,10,11].

In the genus *Flavivirus*, there are several serocomplexes, including DENV, Japanese-encephalitis virus, and tick-borne encephalitis virus serocomplexes. Abs that recognize members from different serocomplexes, members within a serocomplex, or a single member are called flavivirus group-reactive, complex-reactive, or type-specific, respectively [12]. Previous studies of polyclonal human sera revealed that a significant proportion of anti-E Abs after DENV infection was group-reactive and recognized the FL of domain II, whereas only a minor proportion was type-specific and recognized E domain III [13–16]. The change in the amount of anti-FL Abs over time and the role of anti-FL Abs in dengue protection versus pathogenesis remain unclear. Following primary DENV infection, individuals develop monotypic neutralizing Abs against the infecting serotype [9]. A recent study on depletion of human sera by recombinant E protein has shown that cross-reactive Abs (including anti-FL Abs) do not contribute substantially to monotypic neutralization against the infecting serotype after primary DENV infection [17]. After secondary DENV infection, individuals develop not only neutralizing Abs against serotypes to which they have been previously exposed but also cross-reactive neutralizing Abs against serotypes to which they have not yet been exposed (“non-exposed” serotypes) [9]. The nature of such heterotypic neutralizing Abs remains unknown. We hypothesize that the cross-reactive anti-FL Abs may play a role in protection against the non-exposed serotypes after secondary infection.
In this study, we developed a DENV virion-capture ELISA to measure the concentrations of anti-E Abs, [anti-E Abs], against DENV in sera, determined the proportion of anti-FL Abs (% anti-FL Abs) by a previously-described capture ELISA using virus-like particles (VLPs) [14,16], and calculated the concentrations of anti-FL Abs, [anti-FL Abs]. We examined the changes of [anti-E Abs] and [anti-FL Abs] over time in sequential serum samples from 10 cases of primary or secondary DENV infection and then measured the [anti-FL Abs] in 26 additional secondary DENV infections. While [anti-FL Abs] did not correlate with NT_{50} against the current infecting serotype, it correlated with neutralization titers against likely "non-exposed" serotypes in 29 secondary infections. These findings support our hypothesis that anti-FL Abs may play a protective role against the "non-exposed" serotypes after secondary DENV infection.

MATERIALS AND METHODS

Ethics statement and human sera

Thirty-six laboratory-confirmed dengue patients, who were admitted to the Hospital Infantil Manuel de Jesús Rivera in Managua, Nicaragua between October 2006 and October 2008 and followed up for 18 months, were selected arbitrarily for the analysis. The study was approved by the Institutional Review Boards of the University of California, Berkeley, and the Nicaraguan Ministry of Health. Parents or legal guardians of all subjects provided written informed consent, and subjects 6 years of age and older provided assent. DENV infection was confirmed by detection of viral RNA by RT-PCR directed to the capsid region
[18,19]; virus isolation in C6/36 cells [20]; IgM seroconversion between acute and convalescent-phase samples; and/or a \( \geq 4 \)-fold increase in total anti-DENV Abs between acute and convalescent phase samples as measured by Inhibition ELISA [19,21]. Primary DENV infection was defined by an Ab titer by Inhibition ELISA [21] of <10 in acute samples or <2,560 in convalescent (day 14) samples, while secondary infection was defined by an Ab titer by Inhibition ELISA of \( \geq 10 \) in acute samples or \( \geq 2,560 \) in convalescent samples [22]. Disease severity was classified according to the 1997 World Health Organization Guidelines [23].

**Proportion of anti-FL Abs determined by capture ELISA using wild-type (WT) and mutant VLPs**

The plasmids expressing prM/E proteins of DENV1 (Hawaii strain, pCB-D1) and DENV2 (strain 16681) were used to generate WT and mutant VLPs containing mutations at critical FL residues (W101A plus F108A) as described previously [16,24]. VLPs derived from ultracentrifugation of culture supernatants of transfectants of 293T cells were used in a capture ELISA as described previously [14,16]. Briefly, 96-well plates were coated with rabbit anti-serum against DENV1 at 4 °C overnight, followed by blocking with 1% BSA in 16 PBS for 1 hour. VLPs and mutant VLPs (at \(~0.01\ \mu g/ml\) were added, followed by two-fold serial dilutions (using dilution buffer) of each human serum sample, anti-human IgG conjugated to horseradish-peroxidase (HRP), TMB substrate and stop solution [16]. The absorbance at a wavelength of 450 nm (OD 450) with reference wavelength of 650 nm was read. The endpoint titers were calculated
as the reciprocal of the highest titers that yielded a signal greater than 3 standard deviations of the mean signal from multiple (n= 5) normal human sera. The proportion of anti-FL Abs was determined by the formula: % anti-FL Abs = \[1 - \frac{\text{endpoint titer to mutant VLPs}}{\text{endpoint titer to WT VLPs}}\] x 100% [14]. Mixtures of mAbs containing different proportions of a mouse anti-FL mAb (FL0231) and a mouse anti-E domain III mAb (DA6-7) in the VLP-capture ELISA revealed a linear relationship between the proportion of FL0231 (anti-FL mAb) added and the measured proportion of FL0231 (P =0.003, two-tailed Spearman correlation test) (Figure S1). The limit of detection was 4% based on the experiment of mixing mAbs (Figure S1).

**Concentration of anti-E Abs determined by virion-capture ELISA**

The virion-capture ELISA was performed similarly to the VLP-capture ELISA except that DENV1 (Hawaii strain) virions derived from ultracentrifugation of culture supernatants of infected Vero cells were used as antigen. Briefly, 96-well plates were coated with rabbit anti-serum against DENV1 at 4°C overnight, followed by blocking with 1% BSA in 1x PBS for 1 hour and adding DENV1 virions (at ~0.01 μg/ml). To determine the [anti-E Abs], the virion capture-ELISA was initially performed using two-fold serial dilutions of each serum to identify the dilution that gave rise to OD values within the linear range of the standard curve (from 6.6 to 105.63 ng/ml), based on known concentrations of a human mAb 82.11 that targets the FL epitope (Figure 1A) [25]. Then the virion capture-ELISA was performed using such serum dilution in parallel to mAb 82.11 (from 1.65 to
6760 ng/ml) in duplicates. The OD values were interpolated to determine the [anti-E Abs] in each human serum sample (Figure 1B) (GraphPad Prism 5.0, GraphPad software Inc., CA).

**Neutralization test**

The 50% neutralization titer (NT$_{50}$) was determined using a flow cytometry-based neutralization assay with reporter viral particles (RVPs) of four different DENV serotypes as described previously [26]. Briefly, eight 3-fold dilutions of each serum sample were mixed with DENV RVPs (a GFP DENV reporter replicon packaged by DENV structural proteins C-prM / M-E expressed in trans) [26,27] and incubated with human Raji-DC-SIGNR cells for 48 hours. GFP-positive infected cells were quantified by flow cytometry, and raw data extracted using FlowJo software, version 7.2.5 (TreeStar Software) was graphed in the GraphPad Prism 5.0 program as percent infection versus the log of the reciprocal serum dilution. A sigmoidal dose response curve with a variable slope was then generated to determine NT$_{50}$, the Ab dilution at which a 50% reduction in infection was observed compared to the no-Ab control [26,28]. For patients with secondary DENV infection, the current infecting serotype was identified by RT PCR and/or virus isolation, and the previous infecting serotype(s) were determined based on the neutralization pattern, the epidemiology of dominant DENV serotype circulation in Nicaragua, and the age of the patients [29]. For each patient, the remaining serotypes were considered as likely “non-exposed serotypes”.
Statistical analysis

The two-tailed Mann-Whitney test was used to determine the difference in [anti-FL Abs] and [anti-E Abs] among primary and secondary DENV infections. The one-tailed Spearman correlation test was used to determine the relationship between [anti-FL Abs] and NT_{50} using the program GraphPad Prism 5.0. The two-tailed Spearman correlation test was used to determine the relationship between the proportion of anti-FL mAb FL0231 added and that measured in the VLP-ELISA.

RESULTS

Determination of [anti-E Abs], [anti-FL Abs] and % anti-FL Abs in human sera

We first developed a virion-capture ELISA using known concentrations of a human anti-E mAb, 82.11, to generate a standard curve (Figure 1A) [25]; the OD values derived from human sera in the same capture ELISA were interpolated to determine [anti-E Abs] (Figure 1B). MAb 82.11 bound to the E protein of the four DENV serotypes equivalently, as shown by Western blot analysis and virion-capture ELISA (Figure 1C), which is in agreement with previous reports of its neutralization potency against the four DENV serotypes at comparable concentration [25]. Table 1 summarizes the [anti-E Abs] in sera collected longitudinally 3, 6, 12 and 18 months post-illness from 6 confirmed dengue cases with primary DENV infection and 4 dengue cases with secondary
infection. The [anti-E Abs] ranged from 5.8 to 158.8 μg/ml and 58.8 to 1894.9 μg/ml in primary and secondary DENV infections, respectively.

We next employed a previously-described VLP-capture ELISA using DENV1 WT and mutant VLPs containing representative FL mutations (W101A plus F108A) to determine the % anti-FL Abs in each serum sample [14,16]. As shown in Figure 2A, the % anti-FL Abs in the 3-month post-illness serum of a secondary DENV2 case (ID 237) was 57%. We also examined the same serum by using DENV2 WT and mutant VLPs (W101A plus F108A) in the VLP-capture ELISA, and the % anti-FL Abs was found to be 56%. The % anti-FL Abs determined by DENV1 and DENV2 VLP-capture ELISA was similar in the 6-month and 18-month sera of ID237 and in the sera of other cases as well (Figure S2). This finding is consistent with the notion that the FL residues recognized by anti-FL Abs are highly conserved by different flaviviruses, and therefore the % anti-FL Abs determined by VLPs of different DENV serotypes was similar. We thus used DENV1 VLP-capture ELISA to determine the % anti-FL Abs for all sera in this study. Table 1 summarizes the % anti-FL Abs in sequential serum samples from 10 cases, which ranged from 4 to 63%. After determining the % anti-FL Abs we calculated the [anti-FL Abs], which ranged from 0 to 0.94 μg/ml (Table 1).

**Kinetics of [anti-E Abs] and [anti-FL Abs] over time**

As shown in Figure 3A, the [anti-E Abs] in most cases slightly decreased from 3 months to 6 months after infection except one DHF case, ID 274, that
displayed peak anti-E Abs 6 months after infection. There was no difference in [anti-E Abs] between 6 and 12 months and between 12 and 18 months among primary DENV infections (P = 0.66 and 1, respectively) and secondary infections (P = 0.4 and 0.88, respectively, two-tailed Mann-Whitney test). The [anti-E Abs] was higher in secondary infections than in primary infections (P = 0.02, 0.07, 0.01 and 0.06, at 3, 6, 12 and 18 months, respectively, two-tailed Mann-Whitney test). A similar trend in the [anti-FL Abs] was noted, again except ID 274, where [anti-FL Abs] peaked 6 months after infection (Figure 3B). The [anti-FL Abs] was also significantly higher in secondary as compared to primary DENV infections (P = 0.02, 0.04, 0.04 and 0.03, at 3, 6, 12 and 18 months, respectively, two-tailed Mann-Whitney test). Notably, two cases, ID312 (primary DENV1 infection) and ID265 (primary DENV3 infection), had undetectable anti-FL Abs 12 months after infection.

**Relationship between anti-FL Abs and neutralizing Abs in secondary DENV infections**

We then examined the [anti-E Abs], % anti-FL Abs and [anti-FL Abs] in sera collected 12 months post-illness from another 26 patients with secondary DENV infection (Table S1). Of the total of 30 secondary DENV infections, 13 were classified as DF and 17 as DHF/DSS. No significant difference was observed in the [anti-E Abs], % anti-FL Abs or [anti-FL Abs] at 12 months post-infection between DF and DHF/DSS patients (P = 0.48, 0.79, and 0.43,
respectively, two-tailed Mann-Whitney test), although the number of cases examined was small.

We next measured neutralizing Abs in each serum sample against the four DENV serotypes using a previously described flow cytometry-based assay with DENV RVPs [26]. The NT$_{50}$ in 12-month sera from the 30 secondary DENV infections (15 DENV2, 14 DENV3 and 1 DENV1) are summarized in Table 2. For each patient, the previous infecting serotypes were determined based on the neutralization pattern, the epidemiological history of dominant DENV serotype circulation in Nicaragua, and the age of the patient [29]. The serotypes excluding the current and previous infecting serotype(s) were considered as likely “non-exposed serotypes”.

We then examined the relationship between [anti-FL Abs] and NT$_{50}$ 12 months post-infection in patients with secondary DENV infection. [anti-FL Abs] did not correlate with the NT$_{50}$ against the current infecting serotype (Figure 4A and 4E). However, [anti-FL Abs] did correlate with the NT$_{50}$ against the likely “non-exposed” serotypes (DENV4 and DENV2 and DENV1) for patients with secondary DENV3 infection (P = 0.01, P = 0.03 and P = 0.04, respectively, one-tailed Spearman correlation test) (Figure 4B to 4D). For patients with secondary DENV2 infection, [anti-FL Abs] correlated with the NT$_{50}$ against the “non-exposed” serotypes (DENV4 and DENV3) but not DENV1, likely due to the small sample size of this subgroup (P = 0.03, P = 0.04 and P = 0.13, respectively, one-tailed Spearman correlation test) (Figure 4F to 4H).
DISCUSSION

We and others have previously reported that a significant proportion of anti-E Abs in human dengue-immune sera recognize the highly conserved FL residues in domain II of E protein, whereas a small proportion of anti-E Abs recognize E domain III residues [13–16]. The role of anti-FL Abs in protection against subsequent infections and/or dengue pathogenesis remains unclear. In this study, we first developed capture ELISAs to measure the [anti-E Abs] and [anti-FL Abs] in sera from dengue patients to investigate the kinetics of anti-E Abs and anti-FL Abs over time and found that [anti-E Abs] and [anti-FL Abs] were quite stable across time and were higher in secondary DENV infections than in primary infections. We then examined the relationship between [anti-FL Abs] and NT_{50} in patients with secondary DENV infection. While [anti-FL Abs] did not correlate with NT_{50} against the current infecting serotype, it correlated with NT_{50} against likely “non-exposed” serotypes (DENV4, DENV2 and DENV1) in 14 secondary DENV3 cases and “non-exposed” serotypes (DENV4 and DENV3) in 15 secondary DENV2 cases. These findings suggest that anti-FL Abs may play a protective role against “non-exposed” serotypes after secondary DENV infection.

It is known that after primary DENV infection, individuals develop life-long protection against the infecting serotype, which correlates with the appearance of monotypic neutralizing Abs against the infecting serotype [30–34]. The type-specific anti-E neutralizing Abs rather than the group-reactive anti-FL Abs generated after primary infection are believed to contribute to such monotypic neutralizing activity. This concept was supported by the observation that the
monotypic neutralizing activity in human sera after primary infection was greatly reduced by depleting type-specific binding activity with virions of the infecting serotype but was not substantially reduced by depleting cross-reactive binding activity (including anti-FL Abs) with virions of other serotypes [17]. Consistent with this, we found that [anti-FL Abs] did not correlate with NT50 against the infecting serotype in patients with primary DENV infection (data not shown). The nature of the type-specific anti-E neutralizing Abs, which were initially thought to be those targeting E domain III based on the studies of anti-domain III mAbs [35–38], were recently reported to be those targeting quaternary epitopes spanning adjacent E dimers on the virion or possibly other as yet to be identified epitopes [17,39].

After secondary DENV infection, individuals develop not only neutralizing Abs against serotypes to which they have been previously exposed but also heterotypic neutralizing Abs against serotypes to which they have not yet been exposed [9]. The heterotypic neutralizing Abs are believed to account for heterotypic protection against subsequent infection by non-experienced serotypes and contribute to the very low numbers of hospital admission observed after a third or fourth DENV infection in humans [40] as well as the low rate of viremia after a third DENV infection in monkeys [41–44]. The nature of such heterotypic neutralizing Abs remains unknown. One possibility is that some complex-reactive anti-E Abs contribute to the neutralizing activities against “non-exposed” serotypes after secondary infection. Alternatively, the group-reactive anti-FL Abs generated after secondary DENV infection may contribute to such
neutralizing activity. Our findings that [anti-FL Abs] correlated with NT₅₀ against likely “non-exposed serotypes” in secondary cases suggest that anti-FL Abs contribute significantly to heterotypic neutralizing activity against “non-exposed” serotypes after secondary DENV infection. Nonetheless, it is likely that such heterotypic neutralizing Abs against “non-exposed” serotypes include other non-FL cross-reactive Abs as well.

The epitopes recognized by anti-FL Abs include several key residues such as 101W, 106G, 107L and 108F in the FL of E domain II [16,45], which are highly conserved by different flaviviruses and absolutely conserved by the four DENV serotypes. It is conceivable that during secondary DENV infection, memory B cells recognizing these highly conserved FL residues expand and generate anti-FL Abs with higher avidity through affinity maturation [46]. Consistent with this, studies of human sera after DENV infection, which likely contained a significant proportion of anti-FL Abs, showed that the binding avidity of anti-DENV Abs from secondary infections was higher than that from primary infections [28,47]. Moreover, it was reported recently that cross-reactive memory B cells or plasma cells as well as serum avidity increase greatly during acute secondary DENV infection, with greater reactivity to the previous infecting serotype than the current infecting serotype [28,48]. Future studies involving experiments that remove cross-reactive anti-FL Abs from the sera of secondary DENV infections and examine the neutralizing activity against “non-exposed” serotypes will help to further elucidate the contribution of anti-FL Abs to heterotypic neutralizing activity. It is worth noting that while anti-FL Abs are cross-reactive to all four DENV
serotypes, [anti-FL Abs] did not correlate with NT50 against the current infecting serotype (Figure 4A and 4E). This suggests that type-specific or other non-FL Abs probably dominate the neutralizing activity against the current infecting serotype.

In this study, we used known concentrations of a human anti-E mAb (82.11) as a standard in our quantitative virion-capture ELISA to measure [anti-E Abs] in human sera. mAb 82.11, which recognizes FL residues and is a group-reactive neutralizing Ab against four DENV serotypes [25, data not shown], was used as a reference for anti-E Abs in human sera because group-reactive anti-FL Abs constitute a significant proportion of anti-E Abs in human serum. The possibility that some anti-E Abs had different binding properties to the DENV virion compared with 82.11 and were thus over- or under-estimated in the quantification cannot be completely ruled out. To further validate the use of DENV1 virion- and VLP-capture in determining the [anti-E Abs], % anti-FL Abs and [anti-FL Abs] in this study, we used DENV3 virion- and VLP-capture ELISA systems to determine the [anti-E Abs], % anti-FL Abs and [anti-FL Abs] in 14 DENV3 infection samples, and found a nice correlation with these three values determined by the two systems (DENV1 vs. DENV3: r = 0.91, P<0.0001; r = 0.78, P = 0.0009; and r = 0.74, P = 0.0027, respectively, two-tailed Spearman correlation test) (Figure S3). In addition, we determined the [anti-E Abs], % anti-FL Abs and [anti-FL Abs] of 7 DENV2 infection samples using DENV2 virion- and VLP- capture ELISA systems, yielding a good correlation (DENV1 vs. DENV2: r =
0.9, P = 0.0046; r = 0.96, P = 0.0028; and r = 1, P = 0.0004, respectively, two-tailed Spearman correlation test) (Figure S3).

It is also worth noting that the [anti-E Abs] and [anti-FL Abs] determined were for IgG. To address the possibility that IgM might confound these values, we used a previously described IgM ELISA [20,21] to measure anti-DENV IgM and found IgM was negative for all 36 serum samples at 12 months (data not shown), suggesting that IgM is unlikely to confound the 12-month [anti-FL Abs] determined and thus the correlation with NT50 analyzed in Figure 4. Another concern is that anti-prM Abs might affect the anti-E Abs determined. Previous studies have shown that the level of anti-prM Abs in dengue-immune sera (either primary or secondary DENV infection) was much lower than that of anti-E Abs based on the intensity of prM and E bands in Western blot analysis, where the antigens were derived from virus-infected cell lysates and presumably contained equal molar ratios of prM and E proteins [13,49–51]. We used serial dilutions of known concentrations of anti-prM and anti-E human mAbs together with human serum on the same blot and estimated the level of anti-prM Abs in human serum to be 30 fold less than anti-E Abs (data not shown). Therefore, the amount of anti-prM Abs detected in our virion-capture ELISA is trivial compared with that of anti-E Abs, though the possibility of confounding cannot be completely ruled out. Nonetheless, the [anti-E Abs] thus measured, demonstrating higher concentrations in secondary DENV infections as compared to primary infections, is in agreement with previous reports using other methods such as the plaque reduction neutralization test and endpoint dilution determined by ELISA [9,15,52].
Moreover, the [anti-FL Abs] calculated based on [anti-E Abs] and % anti-FL Abs showed a correlation with NT\textsubscript{50} against likely “non-exposed” serotypes in secondary DENV infections, which is consistent with the historical observations of heterotypic neutralization against “non-exposed” serotypes after secondary DENV infection [9]. In summary, our assay for [anti-FL Abs] provides a simple and quantitative method to study the role of anti-FL Abs in protection against or enhancement of dengue disease.

**ACKNOWLEDGMENTS**

We thank Drs. Martina Beltramello, Federica Sallusto, and Antonio Lanzavecchi at the Institute for Research in Biomedicine in Bellinzona, Switzerland, for providing human mAb 82.11, Dr. Gwong-Jen Chang at the Center for Disease Control and Prevention at Fort Collins for kindly providing rabbit sera for the capture ELISA, and Dr. Han-Chung Wu at the Academia Sinica in Taipei, Taiwan for providing mouse mAb DA6-7.
Figure 1. Measurement of [anti-E Abs] in human sera by capture ELISA. (A) Virion-capture ELISA was performed using serial dilutions of a human mAb 82.11 with known concentrations to generate a standard curve. (B) Sequential human serum samples from a dengue patient were tested simultaneously with the standard and the OD values were interpolated to determine [anti-E Abs]. Data are means with standard deviation of duplicates from one representative experiment of two. (C) The binding specificity of mAb 82.11 was determined by Western blot analysis using cell lysates collected from Vero cells infected with mock, DENV1 (Hawaii strain), DENV2 (NGC strain), DENV3 (H87 strain), DENV4 (H241 strain) or WNV (NY99 strain) [16]. The NT50 [25] and dissociation constant (Kd) of mAb 82.11 are summarized on the right. The viron-capture ELISA of DENV1, DENV2 and DENV3 was performed using serial dilutions of mAb 82.11; the Kd was determined using the program GraphPad Prism S.O. Data are means of duplicates from one representative experiment of two. ND, not done.
A

R² = 0.9967

OD 450 nm

[mAb 82.11] (µg/ml)

B

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C

Mo D1 D2 D3 D4 WNV

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Figure 2. Determination of % anti-FL Abs in serum of a dengue patient by VLP-capture ELISA. (A) Serial dilutions of the serum were subjected to a capture ELISA using DENV1 WT and mutant VLPs containing mutations in the FL epitope (W101A+F108A). The bar graph displaying results from an anti-E ELISA shows that comparable amounts of WT and mutant VLPs were added based on recognition of E by pooled human dengue-immune sera. % anti-FL Abs = [1 – endpoint titer to mutant VLPs/endpoint titer to WT VLPs] x 100%. (B) The same serum was subjected to a capture ELISA using DENV2 WT and mutant VLPs (W101A+F108A). Data are presented as in (A). Data are means with standard deviation of duplicates from one representative experiment of two. For endpoint titers, only means are shown.
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### B

#### #237, 3M

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Figure 3. Kinetics of [anti-E Abs] and [anti-FL Abs] in sera of dengue patients. (A) [anti-E Abs] and (B) [anti-FL Abs] in samples collected 3, 6, 12 and 18 months post-infection. Green closed symbols, patients with primary DENV infection; red open symbols, patients with secondary DENV infection.
Figure 4. Relationship between [anti-FL Abs] and NT$_{50}$ against current infecting and “non-exposed” serotypes in secondary DENV infection. The current infecting serotype, previous infecting serotype(s) and “non-exposed serotypes” were determined as described in Methods. [anti-FL Abs] and NT$_{50}$ against the current infecting serotype (A, E) and “non-exposed” serotypes (B to D, F to H) in patients with secondary DENV3 (A to D) and secondary DENV2 (E to H) infections.
Table 1. Concentration of anti-E Abs and anti-FL Abs and proportion of anti-FL Abs in sequential serum samples from 10 dengue cases.

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Immune status</th>
<th>Current infecting serotype</th>
<th>Sampling time (month$^*$)</th>
<th>[anti-E Abs] (ug/ml)$^a$</th>
<th>% anti-FL Abs (%)$^b$</th>
<th>[anti-FL Abs] (&lt;ug/ml)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>256</td>
<td>primary</td>
<td>D1</td>
<td>3</td>
<td>158.8±37.9</td>
<td>41±4</td>
<td>65.1</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>6</td>
<td>118.4±6.6</td>
<td>49±5</td>
<td>58.0</td>
</tr>
<tr>
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<td>16.7</td>
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<td>primary</td>
<td>D1</td>
<td>3</td>
<td>86.0±6.7</td>
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<td>27.5</td>
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<td>64.2±0.4</td>
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<td>BD$^d$</td>
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<td>2.1</td>
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<td>1.8</td>
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</tr>
</tbody>
</table>

$^a$Primary or secondary DENV infection was determined as described in Methods.

$^b$The current infecting serotype was determined as described in Methods. With the exception of two DHF/DSS cases (274, 233), all others were DF cases. D = DENV.

$^c$Sampling time was determined relative to onset of fever.

$^d$[anti-E Abs], [anti FL Abs] and % anti FL Abs were determined as described in Methods.

$^e$Below the limit of detection (BD). The limit of detection of % anti-FL Abs is 4% as described in Methods.

doi:10.1371/journal.pntd.0002451.t001
## Table 2. Neutralization titers in sera of 30 secondary dengue cases 12 months post-infection.

<table>
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<th>Patient ID*</th>
<th>Disease severityb</th>
<th>Age (year born)</th>
<th>Current infecting serotypec</th>
<th>Previous infecting serotype(s)d</th>
<th>NT&lt;sub&gt;60&lt;/sub&gt;e</th>
<th>[anti-FL Abs] (µg/ml)f</th>
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<td>DENV2</td>
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<td>142</td>
</tr>
<tr>
<td>237</td>
<td>DF</td>
<td>14 (1993)</td>
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<td></td>
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<td>223</td>
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<tr>
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<td>14 (1993)</td>
<td>D1</td>
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<td>DSS</td>
<td>9 (1998)</td>
<td>D2</td>
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<td>1433</td>
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<tr>
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<td>14 (1993)</td>
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<tr>
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<td>D3</td>
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<tr>
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<td>DF</td>
<td>10 (1998)</td>
<td>D3</td>
<td></td>
<td>66</td>
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</tr>
</tbody>
</table>

* Secondary DENV infection was determined as described in Methods.

DF, Dengue Fever; DHF, Dengue Hemorrhagic Fever; DSS, Dengue Shock Syndrome.

Previous infecting serotypes were determined based on the neutralization pattern, the epidemiology of dominant DENV serotype circulation in Nicaragua, and the age of patients [29]. Asterisks indicate the highest NT<sub>60</sub>, which suggests the previous infecting serotypes consistent with the epidemiological history.

NT<sub>60</sub> was determined as described in Methods.

[anti-FL Abs] was determined as described in Methods.

Previous infecting serotype(s) could not be determined.
Figure S1. Determination of % anti-FL Abs in mixtures of mAbs containing different proportions of an anti-FL mAb by VLP-capture ELISA. (A) Increasing amounts of mouse mAb FL0231, which recognizes the FL, was mixed with mouse mAb DA6-7, which recognizes E domain III, such that the proportion of anti-FL mAb increased from 0% to 100%. Different mixtures were subjected to a capture ELISA using DENV1 WT and mutant VLPs containing mutations in the FL epitope (W101A+F108A). The bar graph displaying results of an anti-E ELISA shows that comparable amounts of WT and mutant VLPs were added based on recognition of E by pooled human dengue-immune sera. (B) A linear relationship between the proportion of FL0231 (anti-FL mAb) added and the measured proportion of FL0231 was noted ($P = 0.003$, two-tailed Spearman correlation test). % anti-FL Abs (measured proportion of FL0231) = $[1 – \text{endpoint titer to mutant VLPs/endpoint titer to WT VLPs}] \times 100\%$. Data are means with standard deviation of duplicates from one representative experiment of two. For endpoint titers, only means are shown.
Figure S2. Determination of % anti-FL Abs in sequential serum samples from dengue patients by capture ELISA using DENV1 and DENV2 mutant VLPs. (A, B) Serial dilutions of sera (#237 6 and 18 months post-infection) were subjected to a capture ELISA using DENV1 WT and mutant VLPs containing mutations in FL (W101A+F108A) (A) and DENV2 WT and mutant VLPs (W101A+F108A) (B). The data are presented as in Figure 2. (C) The % anti-FL Abs in sera of another 3 patients (#194, #256 and #274) determined by capture ELISA using DENV1 and DENV2 WT and mutant VLPs. Data are means with standard deviation of duplicates from one representative experiment of two. For endpoint titers, only means are shown.
Figure S3. Relationship of [anti-E Abs], % anti-FL Abs and [anti-FL Abs] determined by virion- and VLP-capture ELISA of DENV1 versus DENV3 or DENV2 systems. (A, B, C) [anti-E Abs] (A), % anti-FL Abs (B) and [anti-FL Abs] (C) in serum samples from 14 DENV3 cases were determined by DENV3 virion- and VLP-capture ELISA and were plotted against those determined by DENV1 virion- and VLP-capture ELISA. (D, E, F) [anti-E Abs] (D), % anti-FL Abs (E) and [anti-FL Abs] (F) in serum samples from 7 DENV2 cases were determined by DENV2 virion- and VLP-capture ELISA and were plotted against those determined by DENV1 virion- and VLP-capture ELISA. DENV3 (H87 strain) and DENV2 (NGC strain) virions were used in the virion-capture ELISA. DENV3 and DENV2 prM/E plasmids producing WT and FL mutant (W101A+F108A) VLPs were used to determine the % anti-FL Abs [24]. Data are means with standard deviation of duplicates from one representative experiment of two.
Table S1. Concentration of total anti-E Abs and anti-FL Abs and proportion of anti-FL Abs in sera of 26 dengue cases 12 months post-infection

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Immune status(^a)</th>
<th>Current infecting serotype(^b)</th>
<th>[anti-E Abs] ((\mu\text{g/ml})^c)</th>
<th>% anti-FL Abs ((%)^c)</th>
<th>[anti-FL Abs] ((\mu\text{g/ml})^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>133</td>
<td>secondary</td>
<td>D2</td>
<td>179.1 ± 21.4</td>
<td>38 ± 2</td>
<td>68.1</td>
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<td>295</td>
<td>secondary</td>
<td>D2</td>
<td>118.9 ± 12.7</td>
<td>37 ± 0</td>
<td>44.0</td>
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<td>D2</td>
<td>163.2 ± 6.5</td>
<td>36 ± 1</td>
<td>58.8</td>
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<td>370.9 ± 26.4</td>
<td>57 ± 1</td>
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<td>24 ± 3</td>
<td>73.5</td>
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<td>D2</td>
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</tr>
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<td>secondary</td>
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<td>169.9 ± 7.1</td>
<td>6 ± 2</td>
<td>10.2</td>
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</tbody>
</table>

\(^a,b\) Immune status and the current infecting serotype were determined as described in Methods.
D=DENV.

\(^c\) [anti-E Abs], [anti-FL Abs] and % anti-FL Abs were determined as described in Methods.
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infection are predominantly cross-reactive and recognize epitopes containing highly conserved residues at the fusion loop of domain II. J Virol 82: 6631–6643.


CHAPTER 5

DISCUSSION
SUMMARY

Dengue is the most prevalent arboviral disease in humans worldwide, and vaccines are the most effective means to control the global dengue burdens. The development of an effective dengue vaccine has progressed slowly despite several decades of efforts, because of the lack of knowledge about immune correlates of protection against DENV during natural infection and the required antigenic targets to induce life-long immunity, as well as the challenge to achieve complete and balanced protection against all four serotypes of DENV.

The goal of this study was to better understand the antibody responses that correlated with protection, specifically to identify the role of TS and GR anti-E Abs in neutralization against exposed and non-exposed serotypes after primary and secondary DENV infections, and the antigenic targets that correlate with protection. Our findings will add to the current knowledge of immune responses during nature DENV infection and provide the information required for the development of dengue vaccines.

It is known that after primary DENV infection, individuals develop life-long protection against the infecting serotype, which correlates with the appearance of monotypic neutralizing Abs against the infecting serotype (1-5). The TS anti-E neutralizing Abs probably account for such monotypic neutralizing activity after primary infection, but the epitopes recognized by these long-lasting, potent neutralizing Abs in human sera remains largely unknown. In the first aim of this study (chapter 2), we sought to define the role of TS anti-E Abs in the neutralizing activities of human sera after primary DENV infection and to identify
their epitopes. Based on the depletion experiments, our results demonstrated that TS anti-E Abs, though constituting only a small proportion of anti-DENV Abs, account for the neutralizing activities against the infecting serotype in polyclonal human sera after primary DENV infection. In contrast, cross-reactive anti-E Abs constituted a large proportion in DENV immune human sera, but had negligible neutralizing activity. These observations were consistent with previous reports (6-9). Moreover, our results suggest that TS neutralizing Abs in human sera recognize a structurally complex epitope expressed on intact virus particles, but not on soluble E proteins, which is in agreement with previous studies of human potent neutralizing TS mAbs (9, 10). This observation raised the concern about current subunit dengue vaccines using soluble E protein or DIII as immunogens. Immunization of soluble E proteins or DIII may not elicit strongly neutralizing TS Abs that recognize the complex quaternary epitopes expressed on virions.

We also employed an ELISA in combination with depletion experiment and limiting dilution assays to determine the proportion and titers of TS Abs in human sera and found that the titers of TS binding Abs had a trend of positive correlation with the neutralizing activity in polyclonal human sera after primary DENV infection. Future studies are needed to verify this observation. Measurement of TS anti-E Abs (after depletion of cross-reactive Abs) may be more convenient than the measurement of neutralization to all four serotypes. The % TS Abs and titers of TS Abs in human sera could be potential and useful parameters as correlates of protection after primary DENV infection. Finally, we explored whether DIII of E protein is targeted by TS anti-E neutralizing Abs in
serum samples, since DIII has been reported to be the main target of mouse TS anti-E mAbs (11-18), and the target of several potent neutralizing, human TS anti-E mAbs isolated from memory B cells after DENV infection (9, 19-22). Our results suggested that DIII was recognized by some TS anti-E Abs in human sera after primary DENV infection, as we observed reduction in neutralizing activity in four out of ten serum samples after depletion of anti-DIII Abs. However, DIII may not be the predominant antigenic targets for TS response in human sera. A recent study reported that TS neutralizing Abs in vaccinee sera receiving monovalent DENV vaccine recognized two residues close to DI/DII hinge region (23).

After secondary DENV infection, individuals develop neutralizing Abs not only against the serotypes to which they have been exposed previously, but also against the serotypes to which they have not yet been exposed (24). The heterotypic neutralizing Abs are believed to account for protection against subsequent infection by non-exposed serotypes and contribute to the very low numbers of hospital admission after a third or fourth DENV infection in humans (25) as well as the low rate of viremia after a third DENV infection in monkeys (26-29). In the second aim of this study, we sought to investigate the role of GR anti-E Abs, the major component of cross-reactive Abs in human sera, on neutralizing activity against non-exposed serotypes after secondary DENV infection. First, we investigated the binding avidity, neutralization potency, and neutralization mechanisms of GR anti-E mAbs generated from individuals after primary or secondary DENV infection. Second, we employed a capture ELISA to
determine the concentration of GR anti-E Abs that target FL epitopes ([anti-FL Abs]) in human sera and examine the relationship between [anti-FL Abs] and neutralization titers against the non-exposed serotypes after secondary DENV infection.

In the studies of human GR anti-E mAbs (chapter 3), we demonstrated that human GR anti-E mAbs derived from patients with secondary DENV infection had stronger neutralization potencies and higher binding avidities than those derived from patients with primary infection. To our knowledge, this is the first report showing that dengue immune status of the host (primary versus secondary DENV infection) affects the quality of cross-reactive Abs. GR anti-E mAbs have been reported to recognize residues in the FL or in both FL and bc loop of E protein DII (30, 31). These residues are highly conserved among different flaviviruses and absolutely conserved among the four DENV serotypes. Cross-reactive memory B cells recognizing these conserved residues generated after primary DENV infection are likely to expand greatly when encountering secondary infection and generate higher avidity GR Abs through affinity maturation (32). This interpretation was also supported by the observations that the avidity of anti-DENV Abs in human sera from secondary infection was greater than that from primary infection (33, 34). Moreover, it was recently reported that cross-reactive memory B cells or plasma cells increased greatly during acute secondary DENV infection (34-36). In addition, our results showed GR mAbs derived from primary infection neutralize DENV mainly at the attachment step, whereas GR mAbs derived from secondary infection can also neutralize DENV at
the post-attachment step, presumably by blocking membrane fusion in the acidic environment of endosome. This was also supported by the observation of stronger binding activity after low pH treatment for GR mAbs derived from secondary DENV infection compared with those derived from primary infection. Such stronger binding activity after low pH treatment may contribute to the ability to neutralize DENV at the post-attachment step.

In the study of 29 human sera from patients with secondary DENV infection from a Nicaraguan cohort (chapter 4), our results demonstrated that [anti-FL Abs] did not correlate with neutralization titers against the current infecting DENV serotype, whereas it correlated with neutralization titers against the non-exposed serotypes. These findings, together with the results from chapter 3, suggested that GR anti-E Abs generated from secondary infection have stronger neutralization potencies and higher binding avidities through affinity maturation, and may contribute to heterotypic neutralizing activity that may play a role in protection against subsequent DENV infection with a different serotype. On the other hand, the observation that that [anti-FL Abs] did not correlate with neutralization titers against the current infecting serotype suggested that TS Abs, or other non-FL Abs are probably responsible for the neutralization against the current infecting serotype. Additionally, we observed that in patients with primary DENV infection, though a small sample size, [anti-FL Abs] did not correlate with neutralization titer against the infecting serotype. This was in line with the results from chapter 2 that TS, rather than cross-reactive
Abs, accounted for the neutralizing activity against the infecting serotype in patients with primary DENV infection.

Our assay of measuring [anti-FL Abs] can be used to examine the role of anit-FL Abs in protection, or enhancement of disease in primary, secondary, or tertiary DENV infection in cohort studies as well as to evaluate the relationship of anti-FL Abs to the development of neutralizing Abs against four serotypes in vaccinee sera during vaccine trials.

Collectively, the observations of this study suggest that after primary DENV infection, TS anti-E Abs account for the neutralizing activity against the infecting serotype whereas cross-reactive Abs were weak or non-neutralizing. After secondary DENV infection, GR anti-E Abs had higher neutralizing potencies due to the high binding avidity; the [anti-FL Abs] correlate with the neutralizing activities against the non-exposed serotypes and may contribute to protection in subsequent infection by the non-exposed serotypes.

**FUTURE STUDIES**

While we have demonstrated that the titers of TS anti-E Abs have a trend of positive correlation with the TS neutralizing activity, the correlation was not significant. We will verify this observation with a larger sample size. To avoid the variation between each ELISA, the titers of TS Abs will be calculated by the O.D. value normalized with that of a human anti-E mAb 82.11, which binds to the E protein of all four serotypes of DENV as well as WNV.
Tetravalent formulation is the main strategy of dengue vaccine development currently. We have demonstrated that after secondary DENV infection, GR anti-E Abs have higher binding avidities and neutralization potencies, suggesting that an alternative approach of sequential heterotypic immunization with nonreplicating dengue candidate vaccines may induce potent neutralizing Abs against all four serotypes. Future studies can be designed to test if sequential heterotypic immunization of VLPs or DNA plasmids expressing prM and E proteins in mice elicits comparable level of potent neutralizing Abs, compared with tetravalent immunization, against all four serotypes measured by FRNT.

We found that the concentration of anti-fusion loop Abs, which represent GR anti-E Abs, in the sera of patients with secondary DENV infection correlated with neutralization titers against the non-exposed serotypes, suggesting that GR anti-E Abs may play a role in protection against the subsequent DENV infection of non-exposed serotype. Future studies can be directed to demonstrate the contribution of cross-reactive Abs (GR and CR) to the neutralizing activities in polyclonal sera from patients with secondary DENV infection. By depletion of GR Abs from DENV immune sera with secondary infection using WNV antigens and measurement of the neutralizing activities by FRNT, we can determine if GR Abs account for the neutralizing activities against non-exposed serotypes. By depletion of CR Abs after removal of GR Abs using antigens of non-exposed DENV serotypes, we can also examine the contribution of CR Abs to the neutralizing activities against non-exposed serotypes.
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