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Dengue virus neutralization by human immune sera: Role of envelope protein domain III-reactive antibody

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ABSTRACT

Dengue viruses (DENV) are the etiological agents of dengue fever (DF) and dengue hemorrhagic fever (DHF). The DENV complex consists of four closely related viruses designated DENV serotypes 1 through 4. Although infection with one serotype induces cross reactive antibody to all 4 serotypes, the long-term protective antibody response is restricted to the serotype responsible for infection. Cross reactive antibodies appear to enhance infection during a second infection with a different serotype. The goal of the present study was to characterize the binding specificity and functional properties of human DENV immune sera. The study focused on domain III of the viral envelope protein (EDIII), as this region has a well characterized epitope that is recognized by strongly neutralizing serotype-specific mouse monoclonal antibodies (Mabs). Our results demonstrate that EDIII-reactive antibodies are present in primary and secondary DENV immune human sera. Human antibodies bound to a serotype specific epitope on EDIII after primary infection and a serotype cross reactive epitope on EDIII after secondary infection. However, EDIII binding antibodies constituted only a small fraction of the total antibody in immune sera binding to DENV. Studies with complete and EDIII antibody depleted human immune sera demonstrated that EDIII binding antibodies play a minor role in DENV neutralization. We propose that human antibodies directed to other epitopes on the virus are primarily responsible for DENV neutralization. Our results have implications for understanding protective immunity following natural DENV infection and for evaluating DENV vaccines.

Introduction

Dengue viruses (DENVs) are emerging, mosquito-borne flaviviruses and the causative agents of dengue fever (DF) and dengue hemorrhagic fever (DHF). The DENV complex consists of four serotypes designated DENV1 through 4. A person infected with DENV develops antibodies that cross react with all four serotypes (Roehrig, 2003). However, the antibodies only provide long-term protection against the serotype responsible for the original infection and people can be infected a second time with a different serotype (Halstead, 2002; Rothman, 2004). Individuals experiencing secondary DEN infections face a greater risk of developing severe disease (Halstead, 2002; Rothman, 2004). A leading theory to explain the greater risk of severe disease with secondary DEN infection is that pre-existing cross reactive antibodies bind to the virus and enhance infection of Fc-receptor bearing cells (Halstead, 2003). Despite the fact that DEN vaccines are entering large scale clinical testing, we know remarkably little about the relationship between the binding properties of DEN antibodies in human immune sera and the functional outcome of these interactions.

The major target of flavivirus neutralizing antibody is the Envelope (E) protein, although membrane protein (M) and non-structural protein 1 (NS1) antibodies have also been shown to be protective (Roehrig, 2003; Schlesinger, Brandriss, and Walsh, 1987; Vázquez et al., 2002). E protein is responsible for viral attachment to host cells and the low pH fusion of viral and host cell membranes. The crystal structures of E of several flaviviruses have been solved (Modis et al., 2003; Modis et al., 2005; Nybakken et al., 2006; Rey et al., 1995). Individual subunits of E consist of three beta-barrel domains designated E domains I (EDI), II (EDII) and III (EDIII). Native E is a homodimer that lies flat on the surface of the viral membrane.

Our current understanding of the interactions between DENV and antibody is largely based on studies with mouse monoclonal antibodies (Mabs). DENV neutralizing mouse Mabs have been mapped to all three domains of E. In general, strongly neutralizing mouse Mabs are DENV serotype-specific and bind to an epitopes on EDIII that is unique to each serotype (Crill and Roehrig, 2001;

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Gromowski and Barrett, 2007; Lin et al., 1994; Lok et al., 2008; Roehrig, Bolin, and Kelly, 1998; Sukupolvi-Petty et al., 2007). A DENV type specific epitope on EDIII bound by strongly neutralizing Mabs has been mapped to 4 loops on the lateral face of EDIII (Gromowski and Barrett, 2007; Gromowski, Barrett, and Barrett, 2008; Sukupolvi-Petty et al., 2007). Investigators have also mapped flavivirus cross reactive epitopes on EDIII (Gromowski, Barrett, and Barrett, 2008; Sukupolvi-Petty et al., 2007). Unlike DENV type specific Mabs, cross reactive Mabs that bind to EDIII have moderate to weak neutralizing activity.

Despite the large body of work with mouse Mabs, remarkably little work has been done to characterize the binding properties of human DENV immune sera and to understand the relationship between human antibody binding and neutralization. Convalescent sera from people and horses naturally infected with West Nile virus (WNV), a related flavivirus, had low levels of EDIII-reactive antibody (Oliphant et al., 2007; Sanchez et al., 2007). In WNV immune sera, EDIII binding antibodies were not primarily responsible for neutralization activity (Oliphant et al., 2007; Sanchez et al., 2007). People who have recovered from DENV infections also develop EDIII-reactive antibodies (Beasley et al., 2004; Crill et al., 2009; Hapugoda et al., 2007; Holbrook, Shope, and Barrett, 2004; Ludolfs et al., 2002); however, most human antibody appears to be directed towards a flavivirus cross reactive epitope close to the fusion loop in EDII of DENV (Crill et al., 2009; Lai et al., 2008). To date, no studies have been done to directly test if EDIII-reactive antibodies are primarily responsible for the neutralizing activity of human DENV immune sera. The goal of this study was to measure the level and specificity of EDIII-reactive antibodies in people who have recovered from primary and secondary DENV infections and to determine the contribution of EDIII-reactive antibodies to DENV neutralization.

Results

Dengue immune human sera were obtained by collecting blood samples from volunteers who might have been infected during foreign travel. Of 35 subjects enrolled in the study, 17 had antibodies that neutralized one or more DENV serotypes. The neutralization patterns of the 17 immune subjects were consistent with past exposures to DENV1 only (one subject), DENV2 only (four subjects), DENV3 only (four subjects) and secondary DENV infections (eight subjects). These sera were also tested for DENV neutralizing antibody by the Centers for Disease Control (CDC) in Fort Collins, CO and the Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases (NIAID), Bethesda, Maryland. The CDC and NIAID laboratories reached the same conclusions as we did about the past infection history of these subjects (unpublished data from Drs Robert Lanciotti, CDC and Steve Whitehead, NIH). For the current study we selected 6 sera representing 2 subjects each who had recovered from primary DENV2, primary DENV3 and secondary DENV infections. The DENV neutralization titers and the most likely year and place of infection of these subjects are listed in Table 1.

DENV binding antibodies in human immune sera

Experiments were performed to measure the binding properties of antibodies in the 6 selected immune sera to purified DENV2 and 3. The immune sera were tested at four fold dilutions starting at 1:50. Antibodies in human DENV immune sera cross reacted with both serotypes indicating that the dominant antibodies after primary and secondary infection are serotype cross reactive (Fig. 1). End point virus binding titers were calculated for the 6 sera (Table 2). As expected, subjects with secondary infections had higher titers than subjects with primary infections (Table 2). These results indicate that an ELISA with whole virus as antigen mainly detects serotype cross reactive antibodies and the assay is not predictive of the neutralization properties of the serum sample or past infection history of the subject.

The DENV particle is made up of envelope (E), membrane (M) and capsid (C) proteins. As E protein is the main target of neutralizing antibody, experiments were done to compare the antibody response to E protein and whole virions. As full length E protein alone is not secreted out of cells, we expressed the soluble ectodomain of E (Es) from DENV3 to be used as an antigen. We used immune serum samples # 003 and 011 from primary DENV3 cases and # 009 and 024 from secondary cases and compared binding to DENV3 and Es from DENV3. Antibodies in human immune sera bound well to both Es and virus particles, but greater binding was observed with virus particles compared to Es (Fig. 2). These results demonstrate that although the ectodomain of E is a dominant target of antibody, virions contain epitopes that are absent in recombinant Es.

Purification and characterization of recombinant DENV envelope protein domain III (EDIII)

Studies with mouse Mabs have demonstrated that most DENV serotype-specific antibodies bind to EDIII (Crill and Roehrig, 2001; Gromowski and Barrett, 2007; Lin et al., 1994; Lok et al., 2008; Roehrig, Bolin, and Kelly, 1998; Sukupolvi-Petty et al., 2007). When using whole virus antigen in an ELISA, the cross reactive antibodies in human immune sera are likely to dominate and mask signal originating from serotype-specific antibodies. To develop an assay for measuring serotype-specific antibody, recombinant EDIII was expressed as a MBP fusion protein in *Escherichia coli* (Fig. 3). Previous studies have demonstrated that EDIII expressed alone or as a MBP fusion protein is folded correctly and displays antibody epitopes present on the virion (Maillard et al., 2008; Volk et al., 2004; Volk et al., 2007; Yu et al., 2004). To confirm that recombinant DENV2 and 3 MBP-EDIII fusion proteins produced in our laboratory were correctly folded, binding assays were performed with eight mouse Mabs that bind to EDIII of DENV2 and/or 3. Mabs 3H5-1, 9F16 and 2Q1899 are antibodies that bind to serotype-specific epitopes on the lateral ridge of DENV2 (Gromowski and Barrett, 2007; Henchal et al., 1985; Sukupolvi-Petty et al., 2007). As predicted, all three antibodies bound to MBP-EDIII from DENV2 but not DENV3 (Table 3). We used mouse Mabs 8A1, 14A4 and 1H9 which are serotype-specific neutralizing antibodies that bind to EDIII from DENV3 only (Serafin and Aaskov,

Table 1
Human DENV immune sera used in the study.

Sample ID	Likely year and place of infection	Time interval between infection and sample collection	PRNT50 titer[a]				Most probable past infection
			DENV1	DENV2	DENV3	DENV4	
01[b]	Sri Lanka, 1996	9 years	<1:20	1:271	<1:20	1:42	Primary DENV2 infection
13	South Pacific Island, 1997	8 years	1:178	>1:1280	1:65	1:140	Primary DENV2 infection
11	El Salvador, 1998	7 years	1:84	1:124	1:1032	1:169	Primary DENV3 infection
03	Thailand, 2001	4 years	1:30	1:87	1:338	<1:20	Primary DENV3 infection
09	India or Sri Lanka, 2000	5 years	>1:1280	>1:1280	1:290	1:393	Secondary DENV infection
24	Brazil, 1998	7 years	>1:1280	1:640	1:64	1:108	Secondary DENV infection

[a] The plaque reduction 50 % neutralization titer was determined using Vero cells.
[b] DENV serotype 2 was isolated from serum sample in 1996.