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CHARACTERIZATION OF HUMAN MONOCLONAL ANTIBODIES (HUMABS) SPECIFIC TO DENGUE VIRUS NS1 PROTEIN

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ABSTRACT

Background: The dengue virus (DENV) is responsible for dengue fever (DF) and its severe forms, dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). It is the most important mosquito-borne tropical viral disease in the world. The severe forms of dengue disease have been frequently hypothesized to be correlated with high viral titer and secondary infection with heterologous serotypes, the so called antibody dependent enhancement (ADE), primarily caused by anti-E and anti-prM antibodies. Anti-NS1 antibodies have recently become a focus of interest as another possible factor for severe dengue infection due to their cross-reactivity with human molecules which causes some severe symptoms like vascular leakage. However, information about anti-NS1 antibodies, especially antibodies generated from humans is still limited.

Methods: In this study, 4 anti-NS1 HuMAbs generated from our previous study were characterized. The hybridomas were cultured and re-cloned to obtain individual cells secreting HuMAb before proceeding to antibody gene cloning. Human variable heavy and light chains (VH and VL) were isolated from these hybridoma cells and analyzed using the IMGT/V-QUEST database. HuMAbs were purified from the collected culture supernatant for neutralizing activity test (NT). ***Results:*** Most of the studied anti-NS1 used the same parental germline sequence based on V gene of HC, and V and J gene of LC. We found only minor variations located on the signal peptide. However, they differed in IgG subclass, as 2 clones were IgG1 and another 2 clones were IgG3. In addition, the viral replication inhibition study showed significant NT activity of those 4 HuMAbs. The results obtained from this study provide fundamental information about the genetics and neutralizing activity of anti-NS1 HuMAbs, which can be used for further characterization of their enhancement mechanisms, epitope characterization, and cross-reactivity.

Keywords: Human monoclonal antibody, Dengue virus, Non-structural protein 1 (NS1), Germline, Neutralization activity

INTRODUCTION

The dengue virus (DENV), responsible for the most important mosquito-borne viral disease in the world, belongs to the family *Flaviviridae* and genus *Flavivirus* (Perera and Khun, 2008). Dengue is endemic in more than 128 countries, mainly in tropical and sub-tropical areas, where it is transmitted by *Aedes* mosquitoes. The annual dengue infection rate has been reported as approximately 390 million infections worldwide, of which there are 96 million clinical cases, over 500,000 hospitalizations and 25,000 deaths (WHO, 2016). There are four serotypes of DENV,-1, -2, -3 and -4, that cause dengue disease. People experiencing secondary infection by heterologous serotypes appear to be at greater risk for the more severe forms of the disease, dengue hemorrhagic

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fever (DHF) and dengue shock syndrome (DSS). This is thought to be caused by pre-existing antibodies from a primary infection that fail to neutralize the new infecting virus but facilitate viral entry into F_c gamma receptor bearing cells. This phenomenon is known as antibody-dependent enhancement (ADE) (Endy *et al*., 2004; Sasaki *et al*., 2013).

Dengue virus is a single positive-strand RNA virus, approximately 11 kb in length. The DENV genome encodes three structural proteins (C, prM/M, and E) and seven non-structural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5). Among these non-structural proteins, NS1 is a 45-55 kDa conserved glycoprotein containing 352 amino acids similar in all four serotypes. This protein has an important role in viral replication and immune evasion (Beatty *et al*., 2015; Omokoko *et al*., 2014; Poungpair *et al*., 2014). NS1 circulates as an antigen in the blood of infected patients in the form of a soluble hexamer. Recent research indicates that NS1 can bind to C4 protein and prothrombin (Chuang *et al*., 2013). In addition, anti-NS1 antibodies can cross-react with platelets, plasminogens, and endothelial cells. Taken together, NS1 and its antibodies may contribute to thrombocytopenia, coagulopathy, and vascular leakage in the development of DHF and DSS (Chuang et al., 2016; Cheng *et al*., 2015; Sun *et al*., 2015; Cheng *et al*., 2009). However, the mechanism of NS1 pathogenicity is still unclear. The aim of this study was to characterize four hybridoma clones specific to dengue NS1 protein. The clones are M1 D25-2 B11C3, M20 D25-4 D4C3, M32 D26-5 A2B12, and M34 D27-1 E8A4. Re-cloning of these hybridomas was performed and culture supernatant containing anti-NS1 human monoclonal antibodies (HuMAbs) were collected. Then, these HuMAbs were tested for their viral replication inhibition in Vero cells, and their antibody genes were identified and analyzed by IMGT/V-QUEST database (Brochet *et al*., 2008) This study will be useful information for further characterization such as enhancement mechanism, epitope mapping and analysis of cross-reactivity.

MATERIALS AND METHODS

Cell, virus and HuMAbs

Vero cells, derived from African green monkey kidney cells, were cultured in Minimum Essential Medium (MEM) (Hyclone, USA) supplemented with 10% FBS in a 37°C with 5% CO_2 incubator.

DENV-1 (Mochizuki strain), DENV-2 (New Guinea C (NGC) strain), DENV-3 (H87 strain) and DENV-4 (H241 strain) were propagated in C6/36 cells and cultured at 28°C for 5-7 days or until 60-70% of cytopathic effect (CPE) was observed. Culture fluid was then collected and centrifuged at 3,000 rpm, for 10 min, and stored at -80 °C. The number of virus was determined by Focus Forming Unit (FFU).

Anti-NS1 HuMAbs were prepared from hybridoma cells, cultured at 37°C, in a humidified 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, USA) supplemented with 15% fetal bovine serum (FBS) (Hyclone, USA).

Sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting (WB)

DENV-2 infected C6/36 cell lysates were separated by SDS-PAGE under reducing condition. The protein components were electro-transferred to a polyvinylidene difluoride (PVDF) membrane (GE Healthcare, Sweden). The membranes were then washed and blocked with 5% skimmed milk in 0.05% Tris-buffered saline-tween (TBST). After the blocking step, the separated proteins were incubated with anti-NS1 HuMAbs (1:3 – 1:5 dilution) in 5% skimmed milk TBST at 4 °C overnight. After that, the membrane strips were washed 3 times with 0.05% TBST. Secondary antibody (anti-human IgG (H+L)-HRP conjugated), diluted at 1:10,000 in blocking buffer was then added, and incubated at room temperature for 1 hr. The membrane strips were washed 3 times with washing buffer, before developing the color with a ECL Prime Western blotting detection reagents set (GE Healthcare, USA) and visualized by ImageQuant LAS 4000 mini biomolecular imager (GE Healthcare, USA).

Antibody purification

The collected culture supernatants of HuMAbs were pooled and precipitated with 40% ammonium sulfate, by gently stirring overnight. The precipitate samples were then centrifuged at 5,000 rpm, for 20 min, and the pellet was resuspended with binding buffer. The antibody was then purified by AKTA Prime Plus machine using a protein A column (GE healthcare Life Sciences, USA). The concentrations of IgG were measured using a Pierce™ BCA protein assay kit (Thermo Fisher Scientific, USA).

Immunofluorescence assay (IFA)

Vero cells infected with DENV-1 to 4 (MOI = 0.01) were prepared in 96-well cell culture plate (Corning, New York, USA). Briefly, 50 μl of virus solution, adjusted to 0.01 MOI was added to Vero cells, and incubated for 1 hr. Then 100 μl of MEM with 4% FCS was added to the well. The plate was incubated at 37°C for 3 days, and then fixed with 3.7% formaldehyde/PBS, and followed by 0.1% Triton X-100/PBS. The plate was washed with PBS 3 times, and used for IFA, or stored at 4 °C for no longer than 1 month. Culture fluid of each sample was added to the wells (50 μl/well) of the prepared IFA plate, and incubated for 1 hr at 37°C. After incubation with primary antibody, the cells were washed 3 times with PBS and stained with an Alexa Fluor 488 conjugated goat anti-human IgG (Invitrogen) diluted 1: 1,000 in PBS (50 μl/well) that used as secondary antibody. Then, they were washed 3 times with PBS. After washing, 50 μl of PBS were added to the well, then the bound antibodies were visualized by a fluorescence microscope (Olympus DP72, Tokyo, Japan).

Antibody neutralization test

Vero cells were seeded in a 96-well plate at $2.0x10^4$cells/well for 1 day. The cells were rinsed with 1X PBS before infection with DENV-2 at MOI 0.1 (50 μl/well) and incubated at 37°C for 1 hr. After incubation, the virus solution was discarded and the cells were added to 150 μl/well of two fold serial dilution of antibodies and incubated at 37°C for 2 days. To determine the number of virus propagated in the cell culture supernatant, 50 μl/well of each culture fluid was transferred to Vero cells in 96-well plate and incubated at 37°C for 2 hr. After 2 hr incubation, all supernatant was discarded and 100 μl/well of overlaid media was added followed by incubation for 3 days. After that, the cells were fixed with 3.7% formaldehyde and permeabilized by 0.1% Triton X-100. The infected foci were stained with anti-E HuMAb, followed by Alexa 488 goat anti-human IgG Ab (Invitrogen) at a 1:1,000 dilution. The focus numbers were counted and the percentage of focus reduction was calculated by: % Reduction = (Average number of control foci - Average number of foci from each HuMAb) × 100/Average number of control foci.

Antibody gene cloning and germline analysis

VH and VL gene fragments of four anti-NS1 HuMAbs were amplified from the cDNA of hybridoma cells using 26 pairs of human antibody gene specific primers in 0.2 μl of the Expand High Fidelity PCR system (Roche, Singapore) as previously described (Pitaksajjakul *et al*., 2014). PCR products of all fragments of VH and VL, at an approximate size of 500-700 bps for VL and 600-900 bps for VH were then gel-purified by using the QIAquick® gel extraction kit (Qiagen, Germany), and ligated into pGemT-easy plasmids (Promega, USA), by incubation at 4°C overnight. Afterward, the ligation DNA was transformed into the competent *E.coli* strain DH5α using a heat shock method, and cultured at 37 °C for 1 hr. The cell suspension was then centrifuged at 1,100 g, for 10 min, and all bacterial cells were spread onto LB/Amp containing X-gal/IPTG, and incubated at 37 °C overnight. The next day, 2-3 white colonies derived from each antibody fragment were randomly picked to identify the presence of insert using the colony PCR method. Those clones that showed specific amplification of the insert were then cultured for plasmid preparation using the Purelink plasmid miniprep kit (Invitrogen, USA), and sequenced. The inserted sequences of VH and VL were aligned and analyzed using BioEdit v. 7.2.5 (Hall, 1999) and IMGT/V-QUEST database (Brochet *et al*. 2008).

RESULTS

1. Western blotting

All four HuMAbs were previously determined by their target protein by IFA on HEK 293-T cells expressing DENV-2 NS1 protein (Pipattanaboon *et al*., 2013). The target protein of those 4 HuMAbs was further confirmed by western blot with DENV-2 infected C6/36 cells. The specific reaction to NS1 protein was observed on the membrane with the expected band of an approximate size of 45-46 kDa as shown in Figure 1.

2. Immunofluorescence assay (IFA) and antibody neutralization test

Individual anti-NS1 HuMAbs in culture fluids were examined for the reactivity with DENV infected Vero cell by IFA, using anti-E 4G2 mouse MAb as a positive control. Three HuMAbs cross-reacted to DENV-1, DENV-2 and DENV-3, while (M32) D26-5 A2B12 showed cross-reactivity to DENV-1 to DENV-4 (Figure 2).

Purified antibody was prepared from the culture fluid and used for NT assay. The applied antibody concentration was started at 256 μg/ml and 2-fold serial dilution. All HuMAbs showed neutralizing activity by reducing the number of virus detected in the culture fluid after 2 days incubation (Figure 3).

3. Genetic characterization and germline analysis of anti-NS1 HuMAbs

By using antibody specific primers as described, VH and VL chains were specifically amplified with the size of HC approximately at 600-900 bps, and LC at 500-700 bps (data not shown). The sequences were then analyzed by firstly comparing the obtained sequences with sequences of SPYMEG human fusion partner cell (MBL, Japan). Then, the sequences were blasted to identify the similarity with human IgG sequences. Most of the sequences were >90% identical to human antibody gene sequences. The positions of the framework and CDR regions were located based on the Kabat database (Martin, 1996). After analysis by IMGT/V-QUEST, interestingly, it was shown that most of the clones showed almost identical sequences, with similar parental germline (as shown in Table 1), with minor variations in the signal peptide (data not shown). Interestingly, it was shown that 2 clones (M20D25-4 D4C3 and M34D27-1 E8A4) were classified in isotype

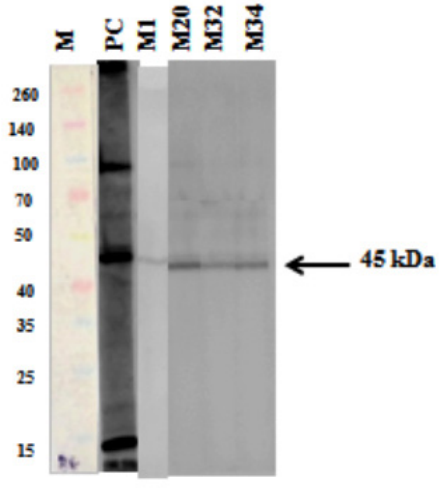

Fig 1- Target protein detection by western blot analysis of 4 HuMAbs. DENV-2 infected C6/36 cell lysates were separated by 12% SDS-PAGE under reducing condition and then, transferred to a PVDF membrane. Blotting membrane was reacted with 4 anti-NS1 HuMAbs (1:5) and then with anti-human HRP antibody (1:10,000). M: molecular mass standards (kDa), PC: human serum positive control, M1: D25-2 B11C3, M20: D25-4 D4C3, M32: D26-5A2B12, M34: D27-1 E8A4.

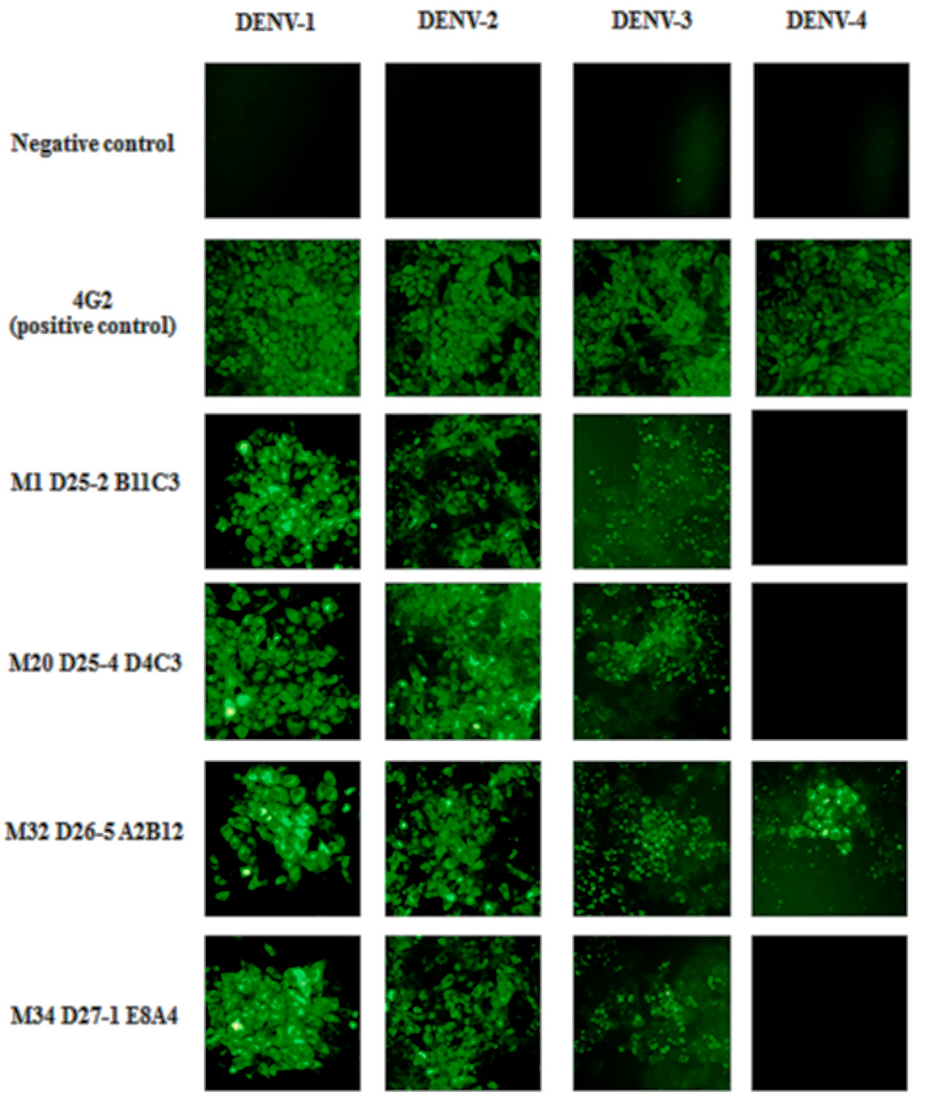

Fig 2- Cross-reactivity of 4 HuMAbs to 4 serotypes of DENV. HuMAbs showed complex and sub-complex binding with DENV-1 to DENV-4 infected Vero cells. PBS was used as negative control and anti-DENV 4G2 MAb was used as positive control.

IgG1, and another 2 clones (M1D25-2 B11C3 and M32D26-5 A2B12) were classified as isotype IgG3 as reported by Omokoko et al., 2014 using a Human IgG Enzyme-Linked Immunosorbent Assay (ELISA) (Omokoko *et al*., 2014).

DISCUSSION

Dengue NS1 and anti-NS1 antibodies from murine MAbs have been reported as a causes of severe dengue pathogenesis (Chuang *et al*., 2013). However, as described by Poungpair *et al*. (2014), anti-NS1 scFv showed *in vitro* and *in vivo* neutralizing activities. These scFv antibodies can inhibit the releases of virus in BHK cells and it can protect the dengue virus inoculated mice after passive administration. In order to clarify the activity and mechanism of action of human anti-NS1, 4 anti-NS1 HuMAbs obtained from our previous study from dengue patients

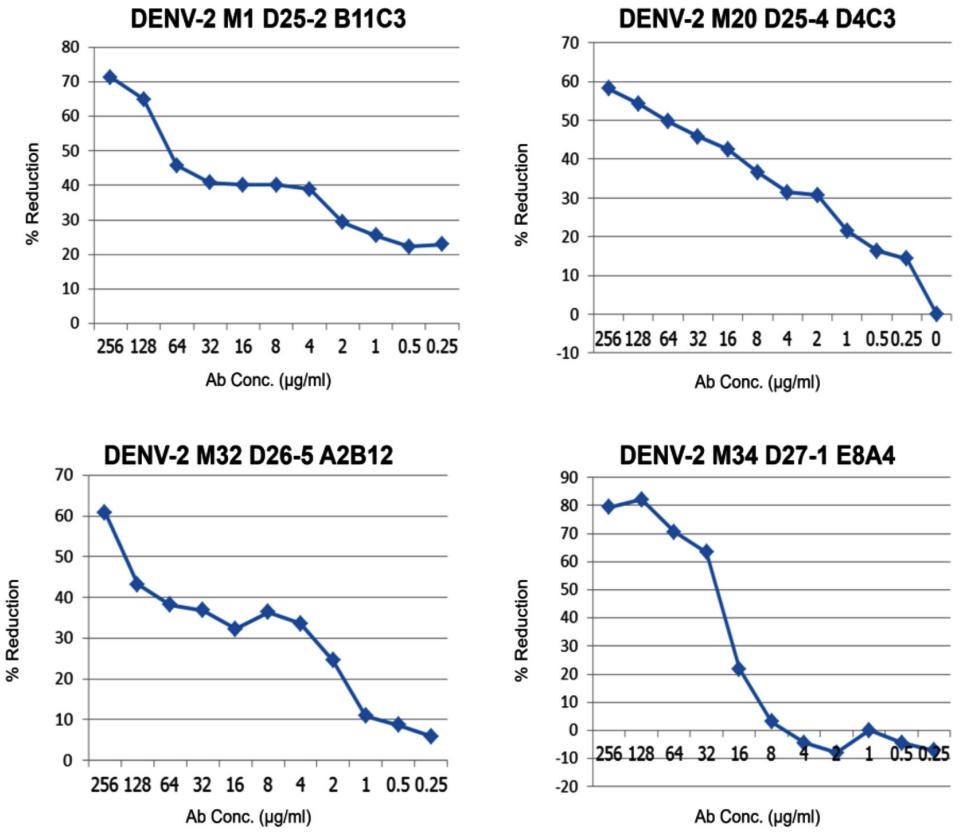

Fig 3- Neutralizing activity of four anti-NS1 HuMAbs. Four HuMAbs showed neutralizing activities in a dose-dependent manner against DENV-2 in Vero cells.

at convalescent phase of the DENV2 secondary infection (Setthapramote *et al*., 2012) were studied for their genetic information and their neutralizing activity.

In terms of binding activity, all anti-NS1 HuMAbs cross-reacted with DENV-1, DENV-2 and DEMV-3 but one HuMAb (M32) showed cross-reactivity with all four serotypes of DENV. NS1 protein is considered to be quite conserved, with Dengue NS1 protein showing 68-80% amino acid similarity among the 4 serotypes (Pushpakumara *et al*., 2016). However, we found that while one HuMAb showed cross-reactivity to 4 serotypes of DENV, the other 3 HuMAbs showed sub-complex activity to bind with only 3 serotypes of DENV. We expected that those complex binding HuMAb should bind to the epitope different from epitope of the other 3 HuMAbs.

In general, antibody genes were diversified by different combinations of VH and VL segments, in which one VH and either Vλ or Vκ germline family, were randomly recombined to generate as individual immunoglobulin (Ujvari *et al*., 2015, Schroeder and Cavacini, 2010). To understand the genetic details of all anti-NS1 HuMAbs, the isolated antibody variable gene and their parental germline of all anti-NS1 HuMAbs were then analyzed by the IMGT/V-QUEST database. Interestingly, most of the clones showed almost identical sequences using the same parental germline. However, we observed that among the 4 clones, 2 of them (M20 D25-4 D4C3, M34 D27-1 E8A4) were classified as subclass IgG1 and 2 clones (M1 D25-2 B11C3, M32 D26-5 A2B12) were subclassed as IgG3. In accordance with previous study, both subclasses (IgG1 and IgG3)

Table 1 Germline analysis using IMGT/V-quest database.

HuMAbs	Iso-types*	Heavy chain			Light chain		% mutation		CDR3 aa sequence (length)	
		IGHV gene	IGHD gene	IGHJ gene	IGKV gene	IGKJ gene	V_H	V_L	HCDR3	LCDR3
M1 D25-2 B11C3	IgG3	HV3-48*02 F	HD3-10*01 F	HJ4*02 F	KV3-20*01 F	KJ2*01 F	8.16	8.16	DRLSSGSGTYYLDY (14)	QQYGFSPPY (9)
M20 D25-4 D4C3	IgG1	HV3-48*02 F	HD3-10*01 F	HJ4*02 F	KV3-20*01 F	KJ2*01 F	8.16	8.16	DRLSSGSGTYYLDY (14)	QQYGFSPPYT(10)
M32D26-5A2B12	IgG3	HV3-48*02 F	HD3-10*01 F	HJ4*02 F	KV3-20*01 F	KJ2*01 F	8.16	9.38	DRLSSGSGTYYLDY (14)	QQYGFSPPYT (10)
M34 D27-1 E8A4	IgG1	HV3-48*02 F	HD3-10*01 F	HJ4*02 F	KV3-20*01 F	KJ2*01 F	8.16	8.16	DRLSSGSGTYYLDY (14)	QQYGFSPPY (9)

*Isotype of each HuMAbs was identified by Human IgG Enzyme-Linked Immunosorbent Assay (ELISA) (Omokoko *et al.*, 2014).

are in a predominant group of secreted human antibodies following viral infection (Vidarsson *et al*., 2014). However, with the same variable region, different in IgG subclass and isotypes can also affect both epitope recognition and antibody affinity (Casadevall and Janda, 2012). As shown in our anti-NS1 HuMAb, even though 4 HuMAbs showed almost identical IgG gene sequences as described, 2 clones of IgG3 subclass (M20 D25-2 B11C3, M32 D26-5A2B12) are complex and subcomplex, respectively, for DENV recognition. Probably, these 2 anti-NS1 antibodies recognized the different epitope in DENV NS1 protein. Further characterization of epitope recognition of these 4 anti-NS1 HuMAbs and their affinity could explain how natural selection of human B cell against NS1 protein is generated in human body. We hypothesize that, to generate anti-NS1 antibodies, usage of VH and VL gene family was focused in a particular germline antibody gene, which is different from our previous study of anti-E HuMAbs (Pitaksajjakul *et al*., 2014). However, more samples of anti-NS1 HuMAbs are required.

We analyzed the neutralizing activity of all anti-NS1 HuMAbs to DENV-2. Unlike anti-E antibodies, anti-NS1 antibodies play an important role in the inhibition of intracellular viral replication (Chuang *et al*., 2013), and viral release (Poungpair *et al*., 2014). Thus, for neutralization activity, we determined the number of viruses released from DENV-infected Vero cells. We found that anti-NS1 antibodies at 256 μg/ml can reduce the virus released from the infected cells by 60-80% comparing with infected cells which is not treated with anti-NS1 antibodies. We suggest that these antibodies can reduce the virus amounts released into culture fluids. This might be related to several mechanisms, e.g. virus entry, replication, assembly and spread, involved with virus morphogenesis (Poungpair *et al*., 2014). For this reason, for the pathogenic role of DENV NS1 to be clarified from the neutralization mechanism, both viral replication inhibition in the presence of complement and cytolytic assay with mediated by antibody-complement complex should be

further studied (Wan *et al*., 2014). As reported by Henchal et al., , a combination of 2 anti-NS1 MAbs showed synergistic activity to protect immunized mice from lethal DENV-2 challenges the same as neutralizing polyclonal antibodies (Hanchal *et al*., 1988). Further study to identify the target epitopes and mechanism of inhibition is required to allow for further application.

The results obtained from this study can lead to greater understanding of the binding and neutralizing activity of anti-NS1 HuMAbs, which can be used for further characterization of their neutralization and enhancement mechanisms, epitope characterization, and their properties of cross-reactivity.

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