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Summary

Mouse antibodies specific to dengue NS1 have been widely studied for their cross-reactivity with several human molecules. This is the first cross-reactivity study of dengue NS1 specific human monoclonal antibodies (HuMAbs), isolated from DENV2 infected patients. Nine anti-NS1 HuMAbs derived mainly from convalescent-phase patients with secondary DENV2 infections were characterized. Their cross-reactivity with plasminogen, thrombin, and endothelial cells was investigated, and then plasmin-formation assays were performed. All anti-NS1 HuMAbs were cross-reactive with human plasminogen (Plg), but not thrombin and endothelial cells. Moreover, all HuMAbs that showed cross-reactivity with Plg converted Plg to plasmin in a plasmin-formation assay. These results suggest the implications and drawbacks of anti-NS1 antibodies for immunotherapy.

INTRODUCTION

Dengue fever is a major public health problem in many countries, especially in tropical and subtropical areas of the world (1). More than 390 million cases of Dengue virus infection are estimated to occur per year (2). Dengvaxia® (CYD-TDV) is the first dengue vaccine, which has been licensed and available in some countries. However, the unequal stimulation of immune response to 4 serotypes caused the limitation of this vaccine (3). Infection with any of the four dengue serotypes (DENV-1, -2, -3, or -4) results in symptoms ranging from mild (dengue fever) to severe (dengue hemorrhagic fever [DHF] and dengue shock syndrome [DSS]). Secondary infection with a different serotype is considered as a major risk factor for severe dengue disease caused by antibody-dependent enhancement (ADE) (4,5). Dengue virus (DENV) is a positive-sense single-stranded RNA virus belonging to the genus Flavivirus in the family Flaviviridae.
The viral genome encodes three structural proteins (capsid [C], pre-membrane [prM], and envelope [E]) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). NS1 is expressed in the host cytosol as a monomer, at the cell membrane as a dimer, and in a secreted form as a hexamer (6). NS1 is known to be immunogenic for the humoral immune response to DENV infection (7). High levels of secreted NS1 (sNS1) have been detected in the sera of patients in the early stage of DENV infection (8), and are produced at high levels during secondary infections (9). The autoimmune effects of anti-NS1 antibodies (Abs) contribute to the pathogenesis of severe dengue (10,11). Several studies have shown that anti-NS1 antibodies in the sera of dengue patients or mouse anti-NS1 monoclonal antibodies (MAbs) cross-react with platelets and endothelial cells, resulting in platelet dysfunction and endothelial cell damage (12,13). Moreover, in patients with severe dengue, anti-NS1 Abs are suggested to be the causative agents of a hemostatic imbalance that may contribute to the hemorrhagic status of patients with DHF/DSS. Nonetheless, the activity of antibodies specific to NS1 that confer protection against DENV infection have been reported in a mouse model (14,15). Since most of reported anti-NS1 antibodies were based on mouse Abs or human polyclonal Abs, in this study, we characterized nine anti-NS1 HuMAbs isolated from DENV-infected patients and investigated their reactivity against human plasminogen, human thrombin, and Microvascular Endothelial Cell line-1 (HMEC-1).

MATERIALS AND METHODS

Viruses and cells: DENV-1 (Mochizuki strain), DENV-2 (New Guinea C or NGC strain), DENV-3 (H87 strain), and DENV-4 (H241 strain) were propagated in Aedes albopictus C6/36 cells as described previously (16). The infective titers were estimated in Vero cells with a focus-
forming assay. Human microvascular endothelial cells (HMEC-1; ATCC® CRL3243™) were cultured in endothelial cell growth medium MCDB 131 (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10 ng/mL Epidermal Growth Factor; EGF (Merck Millipore, Burlington, MA, USA), 1 µg/mL Hydrocortisone (Sigma-Aldrich, St. Louis, MO, USA), 10 mM L-Glutamine (Life Technologies, Grand Island, NY, USA), and fetal bovine serum; FBS (GE Healthcare, South Logan, Utah, USA) to a final concentration of 10%.

**Human MAbs and antibodies:** Anti-NS1 human MAbs were generated by hybridoma technology using PBMCs derived from DENV2 infected patients. The ethic was approved by the institutional ethical review committee of Mahidol University (MUTM 2009-035-02) (16). All antibody-secreting hybridomas were re-cloned with the limiting dilution method to generate individual clones producing an anti-NS1 antibody. The secretion of an anti-NS1 antibody from each hybridoma cell line was confirmed with an immunofluorescence assay (IFA) and western blotting (WB), reacted with DENV-2-infected C6/36 cells. The positive hybridoma clones were expanded and the culture fluids were harvested, centrifuged at 180 × g for 5 min to collect the culture supernatant, and then stored at 4 °C until they were affinity purified. The monoclonal antibodies were purified from the antibody-rich supernatant with HiTrap™ Protein A HP columns and then were concentrated and buffer exchanged with Centriprep 10 kDa (Millipore, USA). Anti-NS1 HuMAb (D27-1E8A4C2; M34) was previously described, and identified as IgG1 subclass (17) This HuMAb was used as subclass control in IgG subclass identification. Dengue patient’s serum derived from acute stage of DENV2 infection was used as control in endothelial cell cross-reactivity study (16).
IFA and Western Blot: African green monkey kidney cells (Vero cells) infected with DENV serotype 1, 2, 3, or 4 or mock infected were collected at 3 days’ post-infection. The cells were fixed with 3.7% paraformaldehyde in phosphate-buffered saline pH 7.4 (PBS) for 30 min, permeabilized with 0.1% Triton X-100 in PBS for 5 min, and incubated with hybridoma culture fluid containing an anti-dengue NS1 HuMAb for 1 h. The cells were then washed three times with PBS and reacted with Alexa-Fluor-488-conjugated anti-human IgG (H+L) antibody (Invitrogen, Carlsbad, CA, USA) for 1 h at room temperature. After the cells were washed three times with PBS, they were observed under a fluorescence microscope (IX71, Olympus, Tokyo, Japan). For WB, the C6/36 cell lysates of uninfected and DENV2-infected cells (at a multiplicity of infection {MOI} of 0.1) and rNS1 protein (the gift from Research Institute for Microbial Disease, Osaka University, Japan) were used as the antigen. The cell lysate suspension or rNS1 were mixed with an equal volume of 2X sample buffer without 2-mercaptoethanol and electro separated with 10% SDS-PAGE. The proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Sigma, Burlington, MA, USA). The membranes were blocked with 5% skimmed milk in washing buffer (0.05% Tris-buffered saline with Tween 20 [TBS-T]) at room temperature for 2 h and then incubated overnight with 0.5–1.0 μg of each HuMAb. After the membranes were washed with washing buffer, they were incubated with horseradish peroxidase (HRP)-conjugated goat anti-human IgG (H+L) antibody (Merck, NJ, USA) for 1 h at room temperature. The interactive signal between the cell lysate and the HuMAb was visualized with the enhanced chemiluminescent ECL™ Western Blotting Detection Reagent (GE Healthcare, Freibrg, Germany) and observed under an ImageQuant™ LAS 4000 Mini image analyzer (GE Healthcare, Freibrg, Germany).
**HuMAb IgG subclass determination:** The IgG subclasses were determined with PCR, as previously described (17), and was determined by enzyme-linked immunosorbent assay (ELISA). In brief, the total RNAs were isolated from the hybridomas with TRIzol Reagent (Invitrogen, CA, USA) and reverse transcribed with an oligo (dT) primer. The resulting complementary DNA (cDNA) was used as the template to amplify each gene. With the appropriate sets of primers, IgG1, IgG2, IgG3, and IgG4 was determined by size of 211-bp, 346-bp, 207-bp, and 210-bp PCR products, respectively. For the ELISA, a 96-well immunoplate was coated with 300 ng of mouse anti-human IgG1 Fc monoclonal antibody (Sigma-Aldrich, St. Louis, MO, USA). After the wells were blocked, hybridoma culture fluid containing an HuMAb was added. In this experiment, anti-NS1 HuMAb M34 IgG1 was used as IgG1 positive control [18]. Anti-E T58IgA that was previously identified by human IgA ELISA quantitation set (Bethyl Laboratories, USA.) and anti-E 54hIgG2 HuMAb that was previously cloned to pFUSE-CHIg-hG2 IgG2 backbone was used as IgG1 non-relavant control. The bound HuMAb was then detected with HRP-conjugated goat anti-human IgG antibody (Merck, NJ, USA). Color development was performed by the addition of 100 μl of 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (Seracare, Milford, USA), and the reaction was stopped by the addition of an equal volume of 1 N o-phosphoric acid. The optical density at 450 nm (OD450) was measured with an ELISA reader (Tecan, Männedorf, Switzerland).

**Endothelial cell-binding assay:** HMEC-1 cells were seeded in a 96-well cell culture plate at a density at 2 × 10^4 cells per well. The cells were fixed overnight with 1% formaldehyde at room temperature and then washed twice with PBS. HuMAbs (10 or 50 μg/ml) or dengue patient’s
serum (1:100 in PBS) were incubated with HMEC-1 cells at room temperature for 1 h. The cells were then washed three times with PBS, and incubated with Alexa-Fluor-488-conjugated goat anti-human IgG antibody at room temperature for 30 min. The binding activity was observed under a fluorescence microscope.

**Cross-reactivity of anti-NS1 HuMAbs with plasminogen and thrombin using ELISA:** Briefly, 300-ng of human plasminogen (Abcam, Cambridge, UK), human thrombin (EMD Chemicals, CA, USA) or BSA (Sigma-Aldrich, MO, USA) were coated on ELISA plate (NUNC, Sigma-Aldrich, USA.). After blocking with 1% BSA/TBST, anti-NS1 HuMAbs (5 μg/ml) or dengue’s patient serum (1:1000), followed by HRP-conjugated goat anti-human IgG (KPL, USA.) were applied. All washing step was performed by 5-times washes with PBST. All samples were then color developed by the addition of 100 μl of TMB substrate, and the reaction was stopped by the addition of an equal volume of 1 N of o-phosphoric acid. The optical density (OD) was measured at 450 nm in ELISA reader (Tecan, Männedorf, Switzerland). The results were recorded as the net OD (OD value of antibody sample minus OD value of diluent without antibody). PBS was used as negative control.

**Plasmin-formation assay:** Plasminogen (50 μg/ml) was incubated with HuMAbs (200 μg/ml) in PBS for 24, 48, or 72 h at 37 °C. The mixture was then analyzed on 10% SDS-PAGE, and stained with 0.25% Coomassie Brilliant Blue. The gel was photographed and analyzed with the ImageJ software. The fold changes in plasmin intensity and plasminogen intensity were normalized to those of plasminogen without antibody. Urokinase (3 U/ml) was used as positive control.
RESULTS

Anti-NS1 HuMAbs: Among several antibody-producing hybridoma cell lines, nine clones secreting anti-NS1 HuMAbs were selected in this study. These HuMAbs were isolated from three patients with secondary DENV-2 infections. Most of them (8/9) were derived from convalescent stage of two DF patients (HuAbs 5, 7, 10, 21, 31, 236, 238, and 241), except one clone that derived from acute phase DHF patients (HuAb 386).

Cross-reactivity of anti-NS1 HuMAbs with four DENV serotypes: The serological reactivity of the anti-NS1 HuMAbs against all four DENV serotypes was determined with IFA. An anti-E HuMAb was used as the positive control to confirm that the Vero cells were infected with each virus. All anti-NS1 HuMAbs reacted strongly with DENV-1 and DENV-2. Most of them showed weak reactivity with DENV-3, and none reacted with DENV-4 (Fig. 1). The reactivity of these HuMAbs against NS1 was confirmed with WB. All HuMAbs recognized both dengue virus infected cell lysate (Fig. 2A) and rNS1 (Fig. 2B) at 42-47 kDa, under non-reducing condition. Our HuMAbs were not observed for any specific binding to uninfected cell lysate (Fig. 2C).

Subclass analysis of the anti-NS1 HuMAbs: All HuMAbs were classified as human IgG1 subclass, determined by both PCR and ELISA. PCR product at 211 bp were obtained from all HuMAbs (data not shown), indicating that our HuMAbs were IgG1 subclass. These results were confirmed with ELISA detected with anti-human IgG1 Fc monoclonal antibody (Fig. 3). IgA (T58IgA) and IgG2 (54hIgG2) human monoclonal antibody were used as negative control.
Detection of HuMAbs binding to HMEC-1

An immunofluorescent assay was performed to determine whether human antibodies against DENV NS1 proteins react with HMEC-1. We found that the reactivity of HuMAbs at 10 μg/ml and HMEC-1 was not observed in all clones. The reactivity at high concentration of anti-NS1 HuMAbs (50 μg/ml), could be observed from HuMAb 7 with weak binding to HMEC-1 (Fig. 4).

HuMAb cross-reactivity with plasminogen and thrombin: We investigated the cross-reactivity of the anti-NS1 HuMAbs with human plasminogen and thrombin, which could affect coagulation and fibrinolysis, respectively. The result showed that all nine clones of anti-NS1 HuMAbs showed cross-reactivity with human plasminogen whereas none of them could bind to human thrombin (Fig 5).

Effect of anti-NS1 HuMAb cross-reactivity with plasminogen on plasminogen activity: To test for plasmin formation, plasminogen was incubated with the different anti-NS1 HuMAbs, and urokinase (as positive control), for 24, 48, or 72 h, followed by an SDS-PAGE analysis. The amount of plasminogen converted to plasmin increased by > 2-fold at 72 h in the presence of anti-NS1 HuMAbs. We found that HuMAb 31 showed the best enhanced plasminogen activity (Fig. 6A). Conversion of plasminogen to plasmin (86 kDa) that induced by urokinase was showed in Fig. 6B.

DISCUSSION

Anti-NS1 antibodies and dengue NS1 have recently been interested as dengue therapeutic or vaccine candidates, because of their absent of ADE. Although anti-NS1 cross-reactive to
several human molecules, that might cause dengue severe symptoms, have been investigated, their protective activity in mice also presented (14,15). Mouse MAbs or human antibodies from dengue patient serum have been previously investigated from several studies for their target epitope, cross-reactivity and protective activity (19,20). This study is the first to characterize cross-reactivity of 9 anti-NS1 HuMAbs, which were mainly derived from convalescence-phase DF samples.

Most of our anti-NS1 HuMAbs were derived from samples collected at convalescent phases of DF patients, except one clone (386) that was derived from a patient with acute DHF. We confirmed the target protein of all clones by western blotting analysis. All of them could bind to a 42-kDa NS1 protein of DENV-2-infected C6/36 cell lysates and the recombinant NS1 (rNS1). All HuMAbs showed similar cross-reactivity to three serotypes of dengue virus (DENV1-3).

We examined the cross-reactivity of the anti-NS1 HuMAbs with host proteins. It is known that the molecular mimicry between dengue NS1 protein and self-antigens may allow host to produce transient autoantibodies. Antibodies against dengue virus NS1 protein that cross-react with platelet, coagulation factor, plasminogen, and endothelial cells can involve thrombopathy, coagulopathy as well as vasculopathy (19). In our study, no anti-NS1 HuMAbs could react with human thrombin, possibly the region of NS1 epitopes-recognized by these HuMAbs may not share epitope with human thrombin.

It has been reported that mouse anti-NS1 antibodies, which recognized the NS1 amino acid 311-330 synthetic peptide could recognized several endothelial cell molecules (22). Also, this anti-endothelial autoantibodies (anti-NS1 antibodies) were previously found in acute phase DHF/DSS higher than in acute phase DF patient. Furthermore, those antibodies that could
reacted with endothelial cell was found in anti-NS1 IgM higher than anti-NS1 IgG, although anti-NS1 IgG titers were higher in natural infection (23). However, in this study, at the maximum concentration, 1/9 anti-NS1 HuMAb (7) which isolated from DF patients showed cross-reactivity with endothelial cell, even though it could not bind to this cell at lower concentration, which usually used by other study (21).

In the fibrinolytic system, plasminogen is converted to plasmin by tissue-type plasminogen activator or urokinase-type. Activated plasmin can degrade fibrin clots. In the present study, we found that all HuMAbs could bind to plasminogen and could induce plasminogen conversion. Chuang has been reported that murine anti-NS1 MAbs with serine protease activity were able to directly convert plasminogen to plasmin, which may cause hyperfibrinolysis and bleeding (24). However, the precise mechanism of the activation of plasminogen by our HuMAbs required further investigation. Considering to our anti-NS1 HuMAbs-induced plasminogen activity, it was interested that most of them were isolated from DF patients, with no or mild symptoms of hemorrhage. One study of plasminogen cross-reactive of E specific antibodies have been reported, and confirmed the correlation of plasminogen cross-reactive with hemorrhage (25). In our case, further study of anti-NS1 antibodies isolated from patients with and without hemorrhage could explain more on this correlation.

Our results indicated that anti-NS1 HuMAbs derived from both acute DHF and convalescent DF DENV infections facilitate the activation of plasminogen, and might involve with disease severity and enhance severity in dengue patients. Hence, more HuMAbs derived from both DF and DHF patients are required. Moreover, fine mapping of critical epitope of all HuMAbs is further needed. As a result, for further application, more studies on effect of
HuMAbs in pathogenesis mechanism is further needed. Thus, the immunopathogenic complication of NS1-induced autoantibodies need to be considered.

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Conflict of Interest: None to declare

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Figure legends

Fig. 1. Reactivity of anti-NS1 HuMAbs with DENV1-4. Vero cells were infected with DENV-1, DENV-2, DENV-3, or DENV-4. The reactivity of each HuMAb with cells infected with each virus was then examined. M34, Anti-E HuMAb and complete medium (Dulbecco’s modified Eagle’s medium) were used as the positive and negative controls, respectively.

Fig. 2. Recognition of native NS1 protein from DENV-2 and rNS1 protein by HuMAbs tested with western blotting. (A) The blot of DENV-2-infected C6/36 cell lysates and (B) rNS1 protein were incubated with 12 HuMAbs. Lanes 1–12 represents anti-NS1 HuMAbs no. 5, 7, 10, 21, 31, 236, 238, 241, 386, anti-NS1 HuMAb (M34), anti-E HuMAbs and anti-prM HuMAbs respectively. All HuMAbs recognized approximately 42 kDa of both DENV-2-infected C6/36 cell lysates and rNS1 protein. Negative control by using Dulbecco’s modified Eagle’s medium was showed in lane 13. (C) HuMAb M34 and 7 were used as candidates for determining reactivity of anti-NS1 HuMAbs with infected and uninfected cell lysate. Lane no. 1 and 2 were loaded with DENV-2-infected C6/36 cell lysates and lane no. 3 and 4 were loaded with uninfected C6/36 cell lysate. Then, lane no. 1 and 3 were reacted to HuMAb M34 and lane no. 2 and 4 were reacted to HuMAb 7.

Fig 3. IgG subclass determination. Hybridoma culture fluids were tested with anti-human IgG1 antibody by ELISA. Error bars indicate standard deviations.
Figure 4. Immunofluorescence assay of HMEC-1 with anti-NS1 HuMAbs. All HuMAbs (10 μg/ml) were not recognized to HMEC-1, whereas clone 7 (50 μg/ml) showed weakly positive to HMEC-1. On the left and right side showed binding activity of HuMAbs at 10 and 50 μg/ml, respectively. Mouse serum and PBS were used as negative control and dengue (DV) patient’s serum was used as positive control.

Figure 5. Binding of HuMAbs to plasminogen and thrombin. Anti-NS1 HuMAbs were incubated with human plasminogen or human thrombin-coated ELISA plates. The reaction was detected as described in Materials and Methods. Error bars indicate standard deviations of two independent experiments.

Figure 6. Effect of plasminogen cross-reactive HuMAbs on plasmin formation. (A) Conversion of plasminogen to plasmin induced by different HuMAbs when incubated at 37 °C for 24, 48, or 72 h. Mixtures were analyzed on SDS-PAGE under reducing conditions. Gels were stained with 0.25% Coomassie Brilliant Blue, and the intensities of the plasminogen and plasmin H bands were computed with ImageJ software. (B) Urokinase (3 U/ml) incubated with plasminogen, as positive control.
