Dengue Virus-Induced Autoantibodies Bind to Plasminogen and Enhance Its Activation

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*J Immunol* 2011; 187:6483-6490; Prepublished online 11 November 2011; doi: 10.4049/jimmunol.1102218

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Dengue virus (DENV) infection can lead to life-threatening dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS) in patients. Abnormal activation of the coagulation and fibrinolysis system is one of the hallmarks associated with DHF/DSS patients. However, the mechanisms that cause pathology in DHF/DSS patients are still unclear. Because conversion of plasminogen (Plg) to plasmin (Plm) is the first step in the activation of fibrinolysis, Abs against Plg found in DHF/DSS patients may be important. Therefore, to investigate the specificity, function, and possible origin of these Abs, we generated several Plg cross-reactive mAbs from DENV-immunized mice. An IgG mAb, 6H11, which recognizes an epitope associated with a dengue envelope protein, demonstrated a high level of cross-reactivity with Plg. The 6H11 Ab was further characterized with regard to its effect on Plg activation. Using Plm-specific chromogenic substrate S-2251, we found that mAb 6H11 demonstrated serine protease activity and could convert Plg directly to Plm. The serine protease activity of mAb 6H11 was further confirmed using serine protease chromogenic substrate S-2288. In addition, we found several Plg cross-reactive mAbs that could enhance urokinase-induced Plg activation. Lastly, mAb 6H11 could induce Plm activity and increase the level of D-dimer (a fibrin degradation product) in both human and mouse platelet-poor plasma. Taken together, these data suggest DENV-induced Plg cross-reactive Abs may enhance Plg conversion to Plm, which would be expected to contribute to hyperfibrinolysis in DHF/DSS patients.

Received for publication August 2, 2011. Accepted for publication October 19, 2011.

This work was supported by Taiwan National Science Council Grants NSC99-2321-B-006-008 and NSC99-2320-B-006-017-MY3 and by the Center of Infectious Disease and Signaling Research, National Cheng Kung University (Tainan, Taiwan).

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Abbreviations used in this article: DENV, dengue virus; DHF, dengue hemorrhage fever; DSS, dengue shock syndrome; Fbg, fibrinogen; HCV, hepatitis C virus; Plg, plasminogen; Plm, plasmin; PPP, platelet-poor plasma.

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www.jimmunol.org/cgi/doi/10.4049/jimmunol.1102218

Dengue Virus-Induced Autoantibodies Bind to Plasminogen and Enhance Its Activation

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Dengue virus infection can lead to life-threatening dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS) in patients. Abnormal activation of the coagulation and fibrinolysis system is one of the hallmarks associated with DHF/DSS patients. However, the mechanisms that cause pathology in DHF/DSS patients are still unclear. Because conversion of plasminogen (Plg) to plasmin (Plm) is the first step in the activation of fibrinolysis, Abs against Plg found in DHF/DSS patients may be important. Therefore, to investigate the specificity, function, and possible origin of these Abs, we generated several Plg cross-reactive mAbs from DENV-immunized mice. An IgG mAb, 6H11, which recognizes an epitope associated with a dengue envelope protein, demonstrated a high level of cross-reactivity with Plg. The 6H11 Ab was further characterized with regard to its effect on Plg activation. Using Plm-specific chromogenic substrate S-2251, we found that mAb 6H11 demonstrated serine protease activity and could convert Plg directly to Plm. The serine protease activity of mAb 6H11 was further confirmed using serine protease chromogenic substrate S-2288. In addition, we found several Plg cross-reactive mAbs that could enhance urokinase-induced Plg activation. Lastly, mAb 6H11 could induce Plm activity and increase the level of D-dimer (a fibrin degradation product) in both human and mouse platelet-poor plasma. Taken together, these data suggest DENV-induced Plg cross-reactive Abs may enhance Plg conversion to Plm, which would be expected to contribute to hyperfibrinolysis in DHF/DSS patients.

Materials and Methods

Patients’ sera

Sera from Kaohsiung were obtained from 32 dengue patients who were in the acute stage of disease during an outbreak of DENV type 2 infections between August and October 2002 in southern Taiwan. Sera from Vietnam were obtained from 48 DENV-infected infants (mainly DENV type 3) during August 1997 to December 2002 with a clinical diagnosis of DHF, as described previously (16). Sera were also obtained from 20 hepatitis C virus (HCV) patients treated at National Cheng Kung University Hospital (Tainan, Taiwan). Normal human sera were obtained from 14 healthy donors.

Virus preparation and reagents

DENV type 2 PL046 strain was prepared, as described previously (17). For DENV particle precipitation, virus supernatants were mixed with one-third volume of precipitation buffer (28% polyethylene glycol 8000, 8% sodium chloride) and incubated overnight at 4˚C. The mixtures were centrifuged at 10,000 × g for 30 min at 4˚C. The DENV particles in the pellet were reconstituted and dialyzed against 10 mM PBS (pH 8.0). The purified DENV were quantitated by Bradford assay (Bio-Rad, Hercules, CA) and frozen at −20/−70˚C before use. Human Plg was provided by Dr. H.-L. Wu (National Cheng Kung University, Tainan, Taiwan). Human thrombin was purchased from Sigma-Aldrich (St. Louis, MO). Chromogenic substrate S-2251 and S-2288 were purchased from Chromogenix (Milano, Italy). MK peptide (MVDRGWGNGCGLFGK) and LT peptide (LDLPLPLWLPGAT) were synthesized by GL Biochem (Shanghai, China). Control mouse IgG was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

Immunization and generation of mAbs

Five 6-wk-old female BALB/c mice were immunized with 50 μg DENV in CFA i.p. and boosted in PBS three times every 2 wk. Three days before fusion, the mice were injected i.v. with 50 μg Ag in PBS. The hybridomas were generated by ClonaCell-HY kit (Stemcell Technologies, Vancouver, BC, Canada), according to the manufacturer’s procedure. Briefly, the splenocytes were fused with FO cells and selected by hypoxanthine–aminopterin–thymidine-based selection medium. Limiting dilution was performed to obtain single colonies. Supernatants from single colonies were collected and screened for Abs against DENV by ELISA. The isotype of these mAbs was determined by Monoclonal Antibody Isotyping Kit (Santa Cruz Biotechnology, Santa Cruz, CA). To isolate mAbs, hybridomas were injected to pristine-primed BALB/c mice for 7–10 d. IgG and IgM mAbs were purified from ascites by protein G-Sepharose beads (GE Healthcare) or protein L resin (GenScript, Piscataway, NJ), respectively. mAbs were dialyzed against PBS (pH 7.4) and stored at −20˚C.

ELISA

For ELISA, 50 μl Plg (5 μg/ml) or DENV (5 μg/ml) in 0.5 M carbonate/bicarbonate coating buffer (pH 9.6) was coated onto 96-well ELISA plate (GeneDireX, Las Vegas, NV) at 4˚C overnight. The wells were blocking by 1% BSA in PBS, followed by three washes of PBS plus 0.05% Tween 20. Abs or sera were incubated on wells at 37˚C for 1 h, followed by three washes of PBS plus 0.05% Tween 20. In some experiments, phages were

FIGURE 1. Binding of Abs in dengue patients' and immunized mouse sera to Plg and DENV. A, Sera from DENV, HCV patients, and healthy donors were incubated on Plg-coated (left panel) and DENV-coated (right panel) ELISA plates. Bound Abs were detected with HRP-conjugated anti-human IgG Ab, as described in Materials and Methods. B, Sera from DENV-immunized mice binding to Plg using ELISA. Each dot represents one sample. Error bars indicate mean OD ± SD. **p < 0.01, ***p < 0.005 (Student t test). C, Inhibition of DENV-immunized sera binding to Plg by preincubating with different doses of DENV or MK peptide. Results are presented as mean ± SD from three independent experiments.
preincubated with mAb or sera for 1 h, followed by adding into the wells, HRP-conjugated goat secondary Abs against mouse (Zymed, San Francisco, CA) or human (Zymed) IgG/IgM were diluted 5000-fold and incubated at 37°C for 1 h. The color was developed using tetramethylbenzidine (Clinical Science Products, Mansfield, MA) as the substrate, and the absorbance was read at OD 450 nm by a VersaMax microplate reader (Molecular Devices, Sunnyvale, CA).

**Immunofluorescence assay**

For immunofluorescence assay, four serotypes of DENV-infected slides were purchased from Yoo-Hong Biotechnology (Taipei, Taiwan). The 6H11 mAb was diluted to 10 μg/ml and incubated with slides. The unbound mAbs were washed by PBS 10 times, followed by adding Alexa 488-conjugated goat anti-mouse IgG Ab (Invitrogen, Camarillo, CA) (200-fold diluted). The stained cells were observed using an Axioskop 2 plus direct microscope (Zeiss, Oberkochen, Germany).

**Epitope mapping using phage-displayed random peptide library**

To map the epitopes recognized by mAbs, we used a phage-displayed random peptide library kit (PhD, 12-mer; New England Biolabs, Ipswich, MA), as previously described (18). Briefly, mAbs (350 ng) were captured by protein G-Sepharose beads (50% aqueous suspension, 50 μl; GenScript) for 30 min, followed by washing with 1 ml TBS/0.5% Tween 20 (TBST). Phages (2 × 10^11) from original library were incubated with mAb complexes for 30 min, followed by washing with 1 ml TBST 10 times. To increase the specificity of phages binding to mAbs, negative selection with normal mouse IgG was performed at second and third round of panning. Unbound phages from negative selection were further incubated with mAb and washed, as described above. Bound phages were eluted with glycine buffer (pH 2.2) and neutralized by 1 M Tris-HCl (pH 9.1). The phages were further amplified for subsequent rounds of panning. In the end, the specific binding of positive phage clones to mAbs was confirmed by ELISA, and their DNA sequences were analyzed using extracted ssDNA following manufacturer’s suggestion.

**Determination of Plm, serine protease activities, and Plm formation by mAbs in vitro**

To test the effect of mAbs on Plg activation, we performed a colorimetric microplate assay using Plm-specific chromogenic substrate S-2251. In brief, 1 μM Plg was incubated with mAbs (30 or 150 μg/ml) for 1–24 h. In the urokinase-induced plasmin formation assay, urokinase (3 U/ml final concentration; Sigma-Aldrich) was coincubated with mAbs (30 μg/ml). The mixtures were then incubated with 1 mM S-2251 (551.6 μg/ml). The kinetic change of OD at 405 nm was measured. Serine protease activity of mAbs was measured using serine protease chromogenic substrate S-2288. The Abs (30, 150, or 300 μg/ml) were incubated with S-2288 (1 mM, 577.6 μg/ml) with or without the presence of different concentrations of Roche Complete protease inhibitor mixture (Roche Diagnostics, Mannheim, Germany) for 2–24 h. The OD value was detected, as described above.

In the Plm formation analysis, Plg (1 μM, 92 μg/ml) was incubated with mAbs (100 μg/ml) for 24, 48, or 72 h at 37°C. In a competition assay, mAbs were preincubated with different concentrations of LT peptide or DENV for 1 h before being added to the Plg. The mixtures were analyzed by 10% SDS-PAGE, followed by staining with 0.25% Coomassie brilliant blue. The gels were photographed and analyzed by Image J software. The fold changes were represented as plasminogen intensity plasminogen intensity.

**Fbg cleavage analysis**

To determine Fbg cleavage, mAbs (100 μg/ml) were preincubated with Fbg (1 μM) for 36 h at 37°C, and then incubated with Fbg (100 μg/ml) for additional 12 h. The mixtures were analyzed by 10% SDS-PAGE, followed by staining with 0.25% Coomassie brilliant and Western blotting. For Western blotting, Fbg fragments were detected using 1:3000 diluted rabbit anti-Fbg β- and γ-chain polyclonal Ab (GeneTex, San Antonio, TX), followed by a 1:10,000 diluted HRP-conjugated goat anti-rabbit Ig Ab (Sigma-Aldrich).

![FIGURE 2](http://www.jimmunol.org/)

**FIGURE 2.** Characteristics of Plg cross-reactive mAbs generated from DENV-immunized mice. A, Recognition patterns and isotypes of DENV-induced Plg-reactive mAbs. B, Dose-response curves of mAbs binding to Plg as determined by ELISA. Data are presented as mean ± SD from three independent experiments. C, Recognition of different serotypes of DENVs by 6H11 mAb. The binding ability of 6H11 to DENV-infected cells was analyzed by immunofluorescence assay, as described in Materials and Methods (original magnification ×200).

![FIGURE 3](http://www.jimmunol.org/)

**FIGURE 3.** Consensus analysis of epitopes of DENVs, NS1, and Plg recognized by mAb 6H11. Identification of epitopes recognized by mAb 6H11 was described in Materials and Methods. Phagetopes were aligned to E protein of four different serotypes of DENVs, NS1, and Plg. Inverse color black background and white letters indicate complete homology or similar characteristic of amino acids between phages and Ags. Phage 1 and phages 3–10 (A) showed an IPMPW-conserved motif to E protein. Phage 2 (B) showed a LPXPW-conserved motif to E protein.
Measurement of D-dimer formation induced by mAbs in human platelet-poor plasma

To detect D-dimer formation induced by mAbs, platelet-poor plasma (PPP) was collected from the supernatant of citrated blood from individuals with normal platelet number by centrifuge at 2500 \( \times g \) for 15 min. mAbs (400 \( \mu \)g/ml) were incubated with PPP (50 \( \mu \)l) for 1 h at 37˚C. The mixtures were further incubated with human thrombin (0.1 NIH units; 25 \( \mu \)l) at 37˚C for 48 h to catalyze the clot formation and lysis. Supernatants were collected after centrifuge at 14,000 \( \times g \) for 10 min, and D-dimer was detected by commercial ELISA kit (American Diagnostics, Newport Beach, CA).

Measurement of mAb-induced Plm activity and D-dimer formation in mice

To identify the effect of mAb on Plg activation and D-dimer formation in vivo, 6-wk-old BALB/c female mice purchased and maintained at the Laboratory Animal Center of National Cheng Kung University were used. All experiments were approved and performed according to the regulations of the Institutional Animal Care and Use Committee of National Cheng Kung University. In brief, BALB/c mice were injected with 50 \( \mu \)l control IgG or 6H11 mAb (2.5 \( \mu \)g/g body weight) i.v. Blood (400 \( \mu \)l) of mice was collected into tubes that contained 100 \( \mu \)l 3.2% sodium citrate. PPP in blood was harvested by centrifugation at 2500 \( \times g \) for 15 min. For Plm activity assay, 10 \( \mu \)l PPP was incubated with S-2238 (1 mM) for 2 h. The OD value at 405 nm was recorded by VersaMax microplate reader. To determine D-dimer level, competition-based mice D-dimer ELISA kits were used (BlueGene, Shanghai, China).

Statistical analysis

Data were presented as mean ± SD from three independent experiments. Student \( t \) test was used to analyze the significance of the difference between the test and the control groups. In all tests, \( p \leq 0.05 \) was considered significant.
Results
Abs cross-react to Plg in DENV patients’ and immunized mouse sera

To confirm the presence of Plg cross-reactive Abs in dengue patients, patients’ sera from Vietnam (n = 48) as well as Kaohsiung (n = 32) were incubated on Plg-coated microtiter plates. As shown in Fig. 1A, sera from dengue patients (n = 80) exhibited significantly higher levels of Ab binding to Plg and DENV than those from normal healthy donors (n = 14) or HCV patients (n = 20). To more directly demonstrate that Abs produced in response to immunization with DENV can cross-react with Plg, we evaluated the sera of DENV-immunized mice. Normal mouse sera and DENV-immunized sera were diluted (1:10–1:10⁶) and assayed for binding to DENV and Plg. DENV-immunized mouse sera demonstrated Ab binding to both DENV and Plg when compared with nonimmune control mouse serum (Fig. 1B). To further charac-
terize the properties of Abs in DENV-immunized sera that cross-reacted with Plg, we performed a competitive inhibition assay using DENV and DENV E protein peptides. Unlike previous studies that have shown that D4E peptide (E protein aa 100–119) can inhibit binding of dengue patients’ Abs to Plg peptide Plg (13, 15), <20% of Ab binding to Plg was inhibited by a synthetic peptide of E protein aa 96–110 (MK peptide) (Fig. 1C).

Generation and characterization of Plg cross-reactive dengue mAb

To further characterize Plg cross-reactive Abs, we generated 500 DENV mAbs from immunized mice, six of which that cross-reacted to Plg were chosen for further study. As shown in Fig. 2A, these six mAbs belong to IgG or IgM isotypes that could recognize either E or NS1 protein or both (6H11). The Plg-binding abilities of these mAbs were also different (Fig. 2B). mAb 6H11 showed the highest level of binding to Plg (with a relative K_d value of 4.95 × 10^{-9}) when compared with DD1, 6E11, and 2A12 mAbs. In addition, when evaluated in an immunofluorescence assay, mAb 6H11 reacted with cells infected with DENV of four different serotypes, suggesting that mAb 6H11 recognizes an epitope shared by all four different serotypes of DENV. Noninfected cells did not stain (Fig. 2C).

Epitope mapping of Plg cross-reactive mAb 6H11

A 12-mer phage-displayed random peptide library was used to identify the epitope recognized by mAb 6H11. After three rounds of biopanning, we chose 10 single-phage clones for sequencing that could specifically bind to mAb 6H11. Among 10 phage clones, 9 of them showed the same sequence (Fig. 3A). Only phage 2 showed minor difference in sequence to other phage clones (Fig. 3B). Alignment analysis of the sequences in these clones showed a consensus motif (LFPWX) and E protein aa 216–225 of all four serotypes of DENVs as well as aa 686–689 of Plg. In addition, a similar IXXPW motif was also found in NS1 protein aa 264–268.

Inhibition of mAb 6H11 and dengue patients’ sera binding to Plg by phage

Because phages 1 and 3–10 showed the same sequence, we used phage 1 to perform inhibition assays. As shown in Fig. 4A, binding of mAb 6H11 to Plg was inhibited in a dose-dependent manner by phage 1, but not by nonspecific control phage. In addition, Plg binding by Abs in dengue patients’ sera was also specifically inhibited by phage 1 (Fig. 4B).

Plg cross-reactive mAbs induce Plg activation and show serine protease activity

To understand whether Plg cross-reactive mAbs could convert Plg to Plm, Plg was incubated with mAbs, followed by analyzing the Plm activity using Plm substrate S-2251. As shown in Fig. 5A, mAbs 6H11, 7D2, and 8E5 could activate Plg to Plm in a dose-dependent manner within 24 h. mAb 6H11 showed the highest activity. Activation of Plg to Plm by mAbs was also time dependent (Fig. 5B). We did not observe any color formation when mAbs were incubated with Plm substrate S-2251 alone (data not shown). However, both mAbs 6H11 and 7D2 can hydrolyze a broad-spectrum substrate of serine protease (S-2288) in a dose- and time-dependent manner (Fig. 5C). Furthermore, a protease inhibitor could inhibit mAb 6H11 hydrolysis of S-2288 in a dose-dependent manner (Fig. 5D). Because activation of Plg can be induced by urokinase, we tested the mAbs for their ability to enhance urokinase-induced Plg activation. Therefore, urokinase (3 U/ml) was incubated with Plg and S-2251 in the presence or absence of of 30 µg/ml mAbs. As shown in Fig. 5E, mAbs 2A12, 8E5, 7D2, and 6H11 showed the ability to significantly enhance S-2251 hydrolysis when compared with the negative control, indicating that mAb can enhance urokinase-induced Plm formation.

Human Plm conversion, Fbg cleavage, and D-dimer formation induced by Plg cross-reactive mAbs

To further verify Plm formation was induced by Plg cross-reactive mAbs, Plg was incubated with mAbs for 24, 48, or 72 h, followed by SDS-PAGE analysis. As shown in Fig. 6A, the control IgG group showed no significant increase of Plg to Plm conversion over the no-Ab control. However, >2-fold increase of Plm was found in mAb 6H11-treated group. To further confirm Plm activation was induced by mAb, we used DENV and LT peptide (E protein aa 214–225) that inhibited the conversion of Plg to Plm in a dose-dependent manner (Fig. 6B). Because Plm can digest Fbg or fibrin to release different degradation products, we first incubated mAbs and Plg with Fbg, followed by SDS-PAGE analysis and Western blotting. From SDS-PAGE analysis (upper panel of Fig. 6C), there were four major bands in Fbg alone (αα-polymers, αα-chain, Ββ-chain, and γ-chain). In the urokinase-treated group, these bands decreased significantly. This effect was also observed in the mAb 6H11- and 2A12-treated groups. In addition, in Western blot analysis using specific Abs that recognized different chains of Fbg, we found γ dimer and Ββ were dramatically decreased in urokinase-, mAb 2A12-, and 6H11-treated groups. Moreover, several Fbg fragments (labeled as fragments A, B, and C) were formed in the urokinase-treated group as well as in the mAb 6H11-treated group. In addition to Fbg cleavage, we also measured D-dimer formation induced by mAbs in human PPP. PPP were preincubated with mAbs for 1 h, and clot formation was induced by adding human thrombin and incubated at 37°C. The D-dimer produced was measured by ELISA. As expected, the levels of D-dimer were significantly higher in the 6H11 mAb group compared with those in the control IgG group (77.4 ± 3.934 versus 58 ± 5.353 µg/ml, p = 0.025) (Fig. 6D).
Plm activity and D-dimer formation induced by 6H11 mAb in vivo

To characterize the role of 6H11 mAb in vivo, we first confirmed mAb 6H11 could recognize mouse Plg by immunoprecipitation and immunoblot assays (data not shown). Control IgG and 6H11 mAb were then injected into mice i.v. The mice were bled and sacrificed 48 h after injection. As shown in Fig. 7A, the Plm activities in PPP of mice treated with mAb 6H11 were 1.5-fold higher than those treated with control IgG. The levels of D-dimer in PPP of mice treated with mAb 6H11 were also significantly higher than those treated with control IgG (15.52 ± 0.9072 versus 8.668 ± 0.3679 ng/ml, p = 0.0015) (Fig. 7B).

Discussion

Molecular mimicry between DENV and host proteins has been proposed to contribute to the pathogenesis of DHF/DSS (7). Autoantibodies against DENV E, NS1, and prM proteins found in DHF/DSS patients can cross-react with different cells such as endothelial cells and platelets, which can lead to cell damage or platelet activation (15, 19–22). In this study, we found Plg cross-reactive Abs in dengue patients from both Kaohsiung (mainly DENV type 2 infection in adult patients) and Vietnam (mainly DENV type 3 in infants). Therefore, Plg cross-reactive Abs occur in DENV patients regardless of the serotype of the virus or age of the patients. In addition, we can also induce the production of these Plg cross-reactive Abs in DENV-immunized mice.

Previous studies have shown that there are Abs in dengue patients’ sera that can recognize synthetic peptide PL⁺, which represents aa 759–779 of Plg. In addition, we can also induce the production of these Plg cross-reactive Abs in DENV-immunized mice. To further confirm that mAb 6H11 can induce Plg activation, we monitored the formation of Plm by SDS-PAGE. Consistent with our expectation, the amount of Plg converted to Plm was significantly increased in the presence of mAb 6H11. Conversion of Plg to Plm was inhibited when mAb 6H11 was preincubated with DENV. It is known that Plg activation is usually mediated by a serine protease such as tissue plasminogen activator or urokinase. In the studies described above, mAb 6H11 was observed to demonstrate serine protease activity using substrate S-2288, as shown in Fig. 5. Therefore, mAb 6H11 is an abzyme (Ab with enzymatic/catalytic activity). Abzymes have been found in many different pathological conditions such as infections and autoimmune diseases, and they are generally slow catalysts that may exhibit slow release of their products (24). In addition to the catalytic property of mAb 6H11, we also found Plg activation induced by urokinase was strongly enhanced in the presence of mAbs such as 2A12, 8E5, and 7D2. Taken together, these data suggest that DENV infection can induce autoantibodies that can bind to Plg and enhance its activation. However, we cannot rule out that Abs against different regions of DENV, such as the region represented by the MK peptide, may have different effects on Plg activation or Plm activity.

Because fibrinolysis in vivo is tightly regulated, the pathogenic roles of these Plg cross-reactive autoantibodies in vivo deserved further investigation. Many different factors such as plasminogen activator inhibitor and α2-antiplasmin play counter-regulatory roles in Plg activation or Plm activity in vivo (9). Whether mAb 6H11 can interfere with these factors is yet to be determined. Nevertheless, when mAb 6H11 was injected i.v. into mice, we found it could increase Plm activity and D-dimer level in mouse PPP. Thus, DENV-induced Plg cross-reactive Abs may contribute to hyperfibrinolysis, as found in DENV-infected nonhuman primates and DHF/DSS patients (25–27). In addition, it is known that local fibrin clot formation may be implicated in host defense against microbial pathogens (28). Therefore, Plg activation induced by DENV Abs may also be involved in immune evasion of DENV.

In summary, we demonstrated that DENV infection can induce Plg cross-reactive autoantibodies that may enhance Plg activation. This information not only furthers our understanding of the molecular mechanism of hemorrhage in DHF/DSS, it also provides useful information that should help us avoid possible side effects while seeking an effective dengue vaccine (29).

Acknowledgments

We thank Keith A. Krolick for help in the revision of this manuscript and Peo Yen Chang for technical assistance.

Disclosures

The authors have no financial conflicts of interest.

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