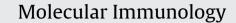
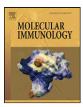
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# Anti-dengue virus nonstructural protein 1 antibodies recognize protein disulfide isomerase on platelets and inhibit platelet aggregation

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# ABSTRACT

Hemorrhagic syndrome is a hallmark of severe dengue diseases. We previously suggested a mechanism of molecular mimicry in which antibodies against dengue virus (DV) nonstructural protein 1 (NS1) crossreact with platelets. In the present study, we demonstrate that protein disulfide isomerase (PDI) on the platelet surface is recognized by anti-DV NS1 antibodies. Anti-DV NS1 obtained from hyperimmunized mouse sera inhibited PDI activity and platelet aggregation, and both inhibitory effects were prevented when anti-DV NS1 antibodies were preabsorbed with PDI. Anti-PDI antibodies bound to a peptide consisting of amino acid residues 311-330 (P311-330) of NS1. This peptide was a predicted epitope analyzed by homologous sequence alignments between DV NS1 and PDI. The platelet binding activities of anti-PDI and anti-DV NS1 antibodies were both reduced by P311–330 preabsorption. Similar to the findings using anti-DV NS1, antibodies against P311-330 bound to PDI and platelets, followed by inhibition of PDI activity and platelet aggregation. Furthermore, the cross-reactivity of dengue hemorrhagic fever patient sera with platelets was reduced when patient sera were preabsorbed with PDI or P311-330. Dengue hemorrhagic fever patient sera also inhibited platelet aggregation, while PDI or P311-330 reduced this inhibitory effect. In conclusion, anti-DV NS1 antibodies cross-react with PDI on platelet surface causing inhibition of platelet aggregation, which may provide implications in dengue disease pathogenesis.

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# 1. Introduction

Dengue virus (DV) infection causes diseases ranging from mild dengue fever to severe dengue hemorrhagic fever or dengue shock syndrome (DHF/DSS) (Henchal and Putnak, 1990; Gubler, 1998; Clyde et al., 2006; Halstead, 2007). A number of mechanisms are involved in the pathogenesis of DHF/DSS progression, including antibody-dependent enhancement of infection (Halstead et al., 1984; Littaua et al., 1990; Mady et al., 1991; Morens, 1994; Anderson et al., 1997; Huang et al., 2006) and host abnormal immune responses to viral infection (Avirutnan et al., 1998; Green et al., 1999; Mathew et al., 1999; King et al., 2000; Lei et al., 2001; Ubol et al., 2008).

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Hemorrhagic syndrome, a feature of DHF/DSS, is a hematologic abnormality resulting from multiple factors, including thrombocytopenia, coagulopathy, and vasculopathy related with dysfunction of platelets and endothelial cells (Rothman and Ennis, 1999; Green and Rothman, 2006). We and others have suggested a mechanism of molecular mimicry, in which dengue patient sera or antibodies (Abs) directed against DV nonstructural protein 1 (NS1) can crossreact with platelets and endothelial cells (Falconar, 1997, 2007; Lin et al., 2001, 2002, 2003, 2008). Anti-platelet autoantibodies elicited by DV NS1 caused thrombocytopenia and mortality in mice (Sun et al., 2007). We have demonstrated that anti-DV NS1 Abs reduce platelet aggregation (Lin et al., 2008). However, the mechanism of anti-DV NS1-mediated inhibition of platelet aggregation remains unclear.

Protein disulfide isomerase (PDI), traditionally thought to be an endoplasmic reticulum protein, can also be expressed on the cell surface (Turano et al., 2002). PDI is localized to the platelet surface (Chen et al., 1995; Essex et al., 1995; Burgess et al., 2000) and is involved in regulation of integrin-mediated platelet aggregation (Essex and Li, 1999; Essex et al., 2001; Lahav et al., 2002; Manickam

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et al., 2008). Those studies also showed that anti-PDI Abs blocked platelet adhesion and aggregation. In the present study, we demonstrated that platelet membrane PDI can be recognized by anti-DV NS1 Abs. The PDI enzymatic activity and ADP-stimulated platelet aggregation were reduced by anti-DV NS1. The DV NS1 amino acid residues 311–330 (P311–330) represent a dominant epitope sharing sequence homology with the thioredoxin domain of PDI.

### 2. Materials and methods

### 2.1. Antibodies and reagents

PDI protein was obtained from TaKaRa (Japan) or ProSpec-Tany TechnoGene Ltd. (Israel). RNase A and cCMP were purchased from Sigma (St. Louis, MO, USA). Anti-PDI was obtained from Stressgen (Victoria, BC, Canada). Horseradish peroxidase (HRP)-conjugated goat anti-mouse, anti-rabbit and anti-human IgG, and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse and anti-human IgG were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). Synthetic peptides of NS1 amino acid residues 311–330 (P311–330, N'[H]-WCCRSCTLPPLRYRGEDGCW-C'[OH]), amino acid residues 10–21 (P10–21, N'[H]-NKELKCGSGIFI-C'[OH]), amino acid residues 152–162 (P152–162, N'[H]-SLEVEDYGFGV-C'[OH]), and amino acid residues 211–225 (P211–225, N'[H]-KIEKASFIEVKSCHW-C'[OH]) were obtained from Sigma-Genosis (Cashmere Scientific, Taiwan).

# 2.2. Recombinant NS1 protein preparation and antibody generation

DV2 NS1 (New Guinea C strain) cDNA was cloned into the pRSET B vector with  $His_6$  Tag. This plasmid was introduced into *Escherichia coli* BL21(DE3)pLysS strain (Invitrogen, CA, USA). The recombinant proteins were induced by a 2 mM final concentration of isopropyl  $\beta$ -D-thiogalactoside and purified with Ni<sup>2+</sup>-chelating chromatography (Amersham Bioscience, Uppsala, Sweden) in TE buffer (50 mM Tris–HCl, pH 8.0, and 2 mM EDTA) containing 8 M urea. The solubilized purified proteins were then refolded by slowly dialyzing with refolding buffer (1 mM EDTA, 50 mM Tris–HCl, 50 mM NaCl, 0.1 mM PMSF, 2 mM reduced glutathione, and 0.2 mM oxidized glutathione) containing sequentially decreased concentrations of urea.

The plasmid construct expressing Japanese encephalitis virus (JEV) NS1 (NT109 strain), pET-32a(+)-JNS1, was obtained from Dr. S.L. Hsieh (National Yang-Ming University, Taipei, Taiwan) and Dr. Y.L. Lin (Institute of Biomedical Science, Academia Sinica, Taipei, Taiwan). This plasmid was derived from pcDNA3-JNS1 and was transformed into the *E. coli* BL21(DE3)pLysS strain. The recombinant protein expression and purification followed the procedures described.

After purification, proteins were examined using 10% SDS-PAGE. Proteins from SDS-PAGE were excised and homogenized in adjuvant to immunize mice. Purified recombinant NS1 protein or synthetic multiple antigenic peptide (MAP) format of P311–330 ( $50 \mu g$ ) were emulsified in complete Freund's adjuvant for the first intraperitoneal (i.p.) immunization to C3H/HeN mice, and 2 weeks later in incomplete Freund's adjuvant for additional four immunizations every week. Mouse sera were collected three days after the last immunization, and IgG was purified using a protein G-Sepharose affinity chromatography column (Amersham Biosciences).

# 2.3. Patient sera

DHF patient sera were obtained from Dr. N. Hung (Department of Dengue Hemorrhagic Fever, Children's Hospital No. 1, Ho Chi Minh City, Vietnam). The diagnosis of DHF was based on the clinical criteria established by the World Health Organization and the parental or guardian informed consent was obtained (Hung et al., 2004, 2006). Among them, two were infected by DV1, one by DV2, seven by DV3, three by DV4, and two with unknown serotype. One patient with DV4 and one with unknown serotype were primary infection, and the other DHF patients were secondary infection. All sera from DHF patients were collected from 3 to 10 days after fever onset. Sera were collected for one to four times in each individual to give a total of 43 samples out of 15 patients. Dengue virus infections in the patients were studied by viral envelope and membrane (E/M)-specific capture IgM ELISA and/or NS1 serotype-specific IgG ELISA at the Center for Disease Control, Department of Health, Taipei, Taiwan. For the experiments, patient sera from 43 samples were pooled with equal volume from each sample. Sera from ten healthy volunteers were used as controls.

#### 2.4. Extraction of platelet membrane proteins (PMP)

Fifty ml of human peripheral blood from each healthy volunteer were mixed with 13 ml of citrate–citric acid–dextrose buffer (100 mM trisodium citrate, 100 mM citric acid, and 2.45% glucose, pH 6.5). After  $100 \times g$  centrifugation at room temperature for 20 min, the upper layer as platelet-rich plasma was centrifuged at  $1000 \times g$  for 10 min and resuspended in homogenization buffer (320 mM sucrose, 50 mM Tris–HCl, 2 mM EDTA, 2 mM EGTA, 5 mM MgCl<sub>2</sub>, 50  $\mu$ M PMSF, and 20  $\mu$ g/ml leupeptine). After freeze and thaw for three times, the platelet suspension was centrifuged at 55,000  $\times$  g for 60 min at 4 °C. The platelet membrane proteins were dissolved with 300  $\mu$ l of homogenization buffer containing 2% Triton X-100, centrifuged at 55,000  $\times$  g for 30 min at 4 °C, and the supernatant was collected.

# 2.5. Western blotting

Proteins separated by SDS-PAGE were transferred to PVDF membranes (Immobilon-P, 0.45 mm; Millipore, Billerica, MA, USA). The membranes were blocked with 5% skimmed milk and then incubated with anti-DV NS1 (1:2500 dilution), anti-JEV NS1 (1:2500 dilution), anti-JEV NS1 (1:2500 dilution), anti-PDI (1:5000 dilution), or control mouse IgG (1:2500 dilution) at 4 °C overnight. After washing with 0.05% PBS-Tween 20, the membranes were incubated with a 1:5000 dilution of anti-mouse or anti-rabbit IgG at room temperature for 30 min. After washing with 0.05% PBS-Tween 20, the membranes were soaked in ECL solution (Western detection kit; PerkinElmer, Boston, MA, USA) for 1 min and then exposed to BioMax light film (Eastman Kodak, Rochester, NY, USA).

# 2.6. ELISA

The microtiter plates were coated with 1 or  $5 \mu g/well PMP$ , DV NS1, PDI, or synthetic peptides at  $4 \,^{\circ}$ C overnight, and then blocked with 5% skimmed milk for 2 h at 37 °C. After washing with 0.05% PBS–Tween 20, appropriate dilutions of anti-DV NS1, anti-PDI, anti-P311–330, or DHF patient sera were added and incubated for  $4 \,^{\circ}$ C overnight. After three washes with 0.05% PBS–Tween 20, HRP-conjugated anti-mouse or anti-rabbit IgG (1:2000 dilution) or anti-human IgG (1:5000 dilution) was added and the preparations were incubated at 37 °C for 2 h. The preparations were then washed and incubated with 3,3',5,5'-tetramethylbenzidine (TMB). The absorbance was measured using an Emax microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 450 nm.

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