

Proteomic Analysis of Endothelial Cell Autoantigens Recognized by Anti-Dengue Virus Nonstructural Protein 1 Antibodies

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We previously showed the occurrence of autoimmune responses in dengue virus (DV) infection, which has potential implications for the pathogenesis of dengue hemorrhagic syndrome. In the present study, we have used a proteomic analysis to identify several candidate proteins on HMEC-1 endothelial cells recognized by anti-DV nonstructural protein 1 (NS1) antibodies. The target proteins, including ATP synthase β chain, protein disulfide isomerase, vimentin, and heat shock protein 60, co-localize with anti-NS1 binding sites on nonfixed HMEC-1 cells using immunohistochemical double staining and confocal microscopy. The cross-reactivity of anti-target protein antibodies with HMEC-1 cells was inhibited by NS1 protein pre-absorption. Furthermore, a cross-reactive epitope on NS1 amino acid residues 311–330 (P311–330) was predicted using homologous sequence alignment. The reactivity of dengue hemorrhagic patient sera with HMEC-1 cells was blocked by synthetic peptide P311–330 pre-absorption. Taken together, our results identify putative targets on endothelial cells recognized by anti-DV NS1 antibodies, where NS1 P311–330 possesses the shared epitope. *Exp Biol Med* 234:63–73, 2009

Key words: proteomic analysis; autoantibodies; endothelial cell autoantigens; dengue virus

Introduction

Clinical manifestations after dengue virus (DV) infection include biphasic fever, body pain, maculopapular rash, and minor hemorrhage. However, some patients can also develop life-threatening dengue hemorrhagic fever or dengue shock syndrome (DHF/DSS) (1, 2). Several mechanisms are involved in the pathogenesis of DHF/DSS progression, including antibody-dependent enhancement, virus variation, and the host immune response (3–13). Some alternate factors, such as innate immune parameters, hyperthermal factors, conditioning of neutralizing antibody, vector transmission, and physical status of virus in viremic individuals may also play roles in the induction of DHF/DSS (14). Hemorrhagic syndromes of DHF/DSS include thrombocytopenia, coagulopathy and vasculopathy, which are related to the dysfunction of endothelial cells and platelets (15–17).

Endothelial damage and activation were observed in the acute phase of DV infection as evidenced by high levels of von Willebrand factor antigen, soluble cell adhesion molecules (sICAM-1 and sVCAM-1), and circulating endothelial cells in peripheral blood from DHF patients (18, 19). Apoptosis in microvascular endothelial cells from lung and intestine tissues was observed in fatal cases of DHF/DSS (20). Although it has been argued that there is no direct evidence indicating DV infection of endothelial cells *in vivo* (11), the dengue viral antigens had been detected in naturally infected human lung and liver endothelial cells (21) as well as in the mouse model (22). The activation and injury of uninfected endothelial cells may be the result of effects exerted by cytokines and cytotoxic factors generated by other dengue-infected cells (4, 11, 17) or by cross-reactive antibodies to endothelial cells (23–27).

Anti-DV nonstructural protein 1 (NS1) antibodies generated in mice have been shown to cross-react with human fibrinogen, platelets, and endothelial cells (23, 24). The cross-reactivity of dengue patient sera with endothelial

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cells has also been demonstrated. Endothelial cells were more reactive with DHF/DSS patient sera than with dengue fever patient sera, and the endothelial cell binding activity of patient sera was inhibited by pretreatment with DV NS1 (25). Furthermore, anti-DV NS1 antibodies induce endothelial cell apoptosis and immune activation (28, 29). Mice immunized with DV NS1 showed antibody deposition in liver vessel endothelium, and also apoptotic cell death of liver endothelium (30). The occurrence of autoimmune responses in DV infection may have implications for the pathogenesis of dengue hemorrhagic syndrome.

In order to confirm a mechanism of molecular mimicry, we investigated the presence of autoantigens on endothelial cells recognized by anti-DV NS1 antibodies. Several candidate proteins were identified using a proteomic analysis. Both the cross-reactivity of anti-DV NS1 antibodies with target proteins and the cross-reactivity of anti-target proteins with NS1 were confirmed. Furthermore, NS1 amino acid residues 311–330 (P311–330) share sequence homology with target proteins.

Materials and Methods

Preparation of Recombinant NS1. Recombinant DV NS1 and Japanese encephalitis virus (JEV) NS1 proteins were prepared as described previously (28). Briefly, the full-length DV2 (New Guinea C strain) NS1 cDNA was cloned into a pRSET B expression vector (Invitrogen, Carlsbad, CA) to establish a pRSET-DVNS1 plasmid. This plasmid was then introduced into *Escherichia coli* BL21(DE3)pLysS strain (Invitrogen). The recombinant NS1 proteins were induced with 2 mM isopropyl β -D-thiogalactoside and purified with Ni²⁺-chelating chromatography (Amersham Biosciences, Uppsala, Sweden) in TE buffer (50 mM Tris-HCl, pH 8.0, and 2 mM EDTA) containing 8 M urea. A single band was detected by SDS-PAGE, and the protein sequence was confirmed. Single band-containing fractions were collected, and the solubilized purified proteins were then slowly dialyzed with dialysis 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 decreasing concentrations of urea.

The plasmid construct expressing JEV (NT109 strain) NS1, 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 above for DV2 NS1 preparation.

Generation of Anti-NS1 Antibodies. Polyclonal antibodies were obtained from C3H/HeN mice immunized intraperitoneally with purified recombinant DV2 or JEV NS1, as previously described (28).

Patient Sera. Fifteen dengue 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. 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 acute phase. 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 (31). Sera from ten healthy volunteers were used as controls. The source of the healthy volunteers without the presence of anti-dengue antibody was from Tainan, Taiwan.

Cell Culture. Human microvascular endothelial cells (HMEC-1) were grown in culture plates containing Medium 200 (Cascade Biologics, Portland, OR) supplemented with 2% fetal bovine serum, 1 μ g/ml hydrocortisone, 10 ng/ml epidermal growth factor, 3 ng/ml basic fibroblast growth factor, 10 μ g/ml heparin, and antibiotics.

Endothelial Cell Membrane Protein Extraction. HMEC-1 cells were washed in phosphate buffered saline (PBS) and scraped with homogenization buffer (320 mM sucrose, 50 mM Tris-HCl, 2 mM EDTA, 5 mM MgCl₂, 50 μ M PMSF, 20 μ g/ml leupeptin, and 2 mM EGTA). Cells were disrupted in a Dounce homogenizer and centrifuged at 435 g for 10 mins. The supernatant was then centrifuged at 100,000 g at 4°C for 30 mins. The pellet was resuspended in 1% Triton X-100 homogenization buffer and incubated on ice for 50 mins. The endothelial membrane proteins (EMPs) were centrifuged at 100,000 g for 30 mins.

For cell surface biotinylation, the cell suspension was mixed with 10 mM biotin reagent (Pierce, Rockford, IL) and incubated at 4°C for 30 mins. After washing with 10 mM Tris-HCl (pH 7.5) to terminate the reaction, the biotinylated cells were incubated with 1 ml octylglucoside lysis buffer (1% Triton X-100, 60 mM n-octyl β -D-glucopyranoside, 150 mM NaCl, 1 mM EDTA, 25 mM Tris-HCl (pH 7.4), and a protease cocktail) at 4°C for 30 mins. After centrifugation at 135 g for 20 mins, the supernatant was collected on an immobilized streptavidin column (Pierce).

Two-Dimensional Electrophoresis (2-DE). The EMPs were mixed with an 11% TCA/acetone (1:9) solution containing 20 mM dithiothreitol (DTT), and then centrifuged at 10,000 g for 15 mins. The pellet was washed twice in acetone with 20 mM DTT and resolubilized with rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS, 0.5% IPG buffer, 0.002% bromophenol blue, and 20 mM DTT) for isoelectric focusing (pH 4–7). The prepared samples (250 μ l each, 1 mg/ml) were applied to IPG strips (Immobiline DryStrip (pH 4–7), 18 cm; Amersham Pharmacia, Piscataway, NJ), followed by the IPGphor

system (Amersham Pharmacia). The strips were then placed onto 12% SDS-PAGE gels for second dimension separation.

Western Blotting. Proteins separated using 2-DE were transferred to 0.45-mm PVDF membranes (Immobilon-P; Millipore, Billerica, MA). 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-ATP synthase (ATPase; 1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), anti-protein disulfide isomerase (PDI; 1:5000 dilution; Stressgen Biotechnologies, Victoria, BC, Canada), anti-vimentin (1:100 dilution; Sigma-Aldrich, St. Louis, MO), anti-heat shock protein 60 (HSP60; 1:200 dilution; Santa Cruz Biotechnology), 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 horseradish peroxidase (HRP)-conjugated anti-mouse, anti-rabbit, or anti-goat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) at room temperature for 30 mins. The membranes were then soaked in ECL solution (Western Lightning kit; Perkin-Elmer, Boston, MA) for 1 min, and then exposed to BioMax light films (Eastman Kodak, Rochester, NY).

Mass Spectrometry Analysis and Database Searching. In-gel digests of protein spots from silver-stained gels were analyzed using mass spectrometry (LCQ DECA XP Plus Ion Trap Mass Spectrometer; ThermoFinnigan, San Jose, CA). The proteins were identified by their peptide fragmentation pattern using the MS/MS ion search of the Mascot program (<http://www.matrixscience.com/>) in the NCBI and Swiss-Prot protein sequence databases.

Immunostaining for Co-Localization. HMEC-1 cells were seeded in monolayers on sterile glass slides coated with 1% gelatin. The nonfixed HMEC-1 cells on glass slides were incubated with adequately diluted primary antibodies, including mouse anti-DV NS1 plus goat anti-ATPase, rabbit anti-PDI, goat anti-vimentin, or goat anti-HSP60 antibodies at room temperature for 1 hr. After washing three times with PBS, the cells were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG plus either tetramethylrhodamine isothiocyanate (TRITC)-conjugated anti-rabbit or anti-goat IgG (Jackson ImmunoResearch Laboratories) at 4°C for 1 hr, and then washed with PBS. The cells were incubated for another 15 mins with each of the primary and secondary antibodies at room temperature. Antibody binding to endothelial cells was observed by confocal microscopy (TCS SPII; Leica, Heidelberg GmbH, Germany).

Pre-Absorption and Cell Binding Assay. HMEC-1 cells were incubated with anti-JEV NS1, anti-DV NS1, anti-ATPase, anti-PDI, anti-vimentin, anti-HSP60, control IgG, monoclonal anti-DV NS1 antibody 11-H11 (obtained from Dr. Kao-Jean Huang, Department of Microbiology and Immunology, National Cheng Kung University Medical College, Tainan, Taiwan), DHF patient sera or purified IgG from DHF patient sera at room temperature for 1 hr. After washing three times with PBS, the cells were

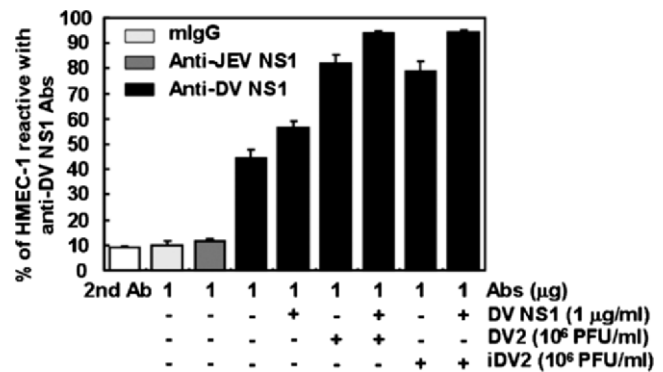


Figure 1. The presence of DV NS1 or virus enhances the endothelial cell binding activity of anti-DV NS1 antibodies. HMEC-1 cells were pre-incubated with 1 μg/ml of DV NS1 protein, 10⁶ PFU/ml of DV2 or heat inactivated DV2 (iDV2), and then incubated with 1 μg of anti-DV NS1 antibodies. The control mouse IgG and anti-JEV NS1 were used as the negative controls. The antibody binding ability to HMEC-1 cells was then analyzed using flow cytometry. Results are shown as mean ± SD from triplicate cultures.

incubated with FITC-conjugated anti-mouse, anti-rabbit, anti-goat, or anti-human IgG for 1 hr at 4°C and analyzed using flow cytometry (FACSCalibur; Becton Dickinson Biosciences, San Jose, CA). To determine the effect of DV NS1 or DV2 PL046 strain on antibody binding to endothelial cells, HMEC-1 cells were pre-incubated with 1 μg/ml of DV NS1 or 10⁶ PFU/ml of DV or heat inactivated DV (56°C, 30 mins) for 1 hr at 37°C. After three washes with PBS, the cells were incubated with anti-DV NS1 at room temperature for 1 hr, followed by FITC-conjugated anti-mouse IgG for 1 hr at 4°C and analyzed using flow cytometry. Pre-absorption was performed using a solid-phase capture technique. An ELISA microtiter plate was coated with DV NS1 or synthetic peptides with 100 μl/well of 10 μg/ml and blocked using 5% skimmed milk. Antibody samples were added to the plate for absorption at 4°C overnight, and then centrifuged at 13,200 g to remove large complexes. The supernatant was collected from each well and incubated with HMEC-1 cells for a cell binding assay. Synthetic peptides, P311–330 (residues 311–330, N'[H]-WCCRSCTLPPLRYRGEDGCW-C'[OH]) and P211–225 (residues 211–225, N'[H]-KIEKASFIEVKSCHW-C'[OH]), were obtained from Sigma-Genosys (Cashmere Scientific, Taiwan).

ELISA. The microtiter plates were coated with 1 μg/well DV NS1, PDI, vimentin or HSP60, left at 4°C overnight, and then blocked with 5% skimmed milk for 2 hrs at 37°C. After washing with 0.05% PBS-Tween 20, appropriate dilutions of primary antibodies were added and incubated at 4°C overnight. Following a 0.05% PBS-Tween 20 wash, HRP-conjugated anti-mouse, anti-rabbit, or anti-goat IgG (1:2000 dilution) was added and the preparations were incubated at 37°C for 2 hrs. The preparations were then washed and incubated with 3,3',5,5'-tetramethylbenzidine (TMB) or 2,2'-azino bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), and the absorbance was measured using

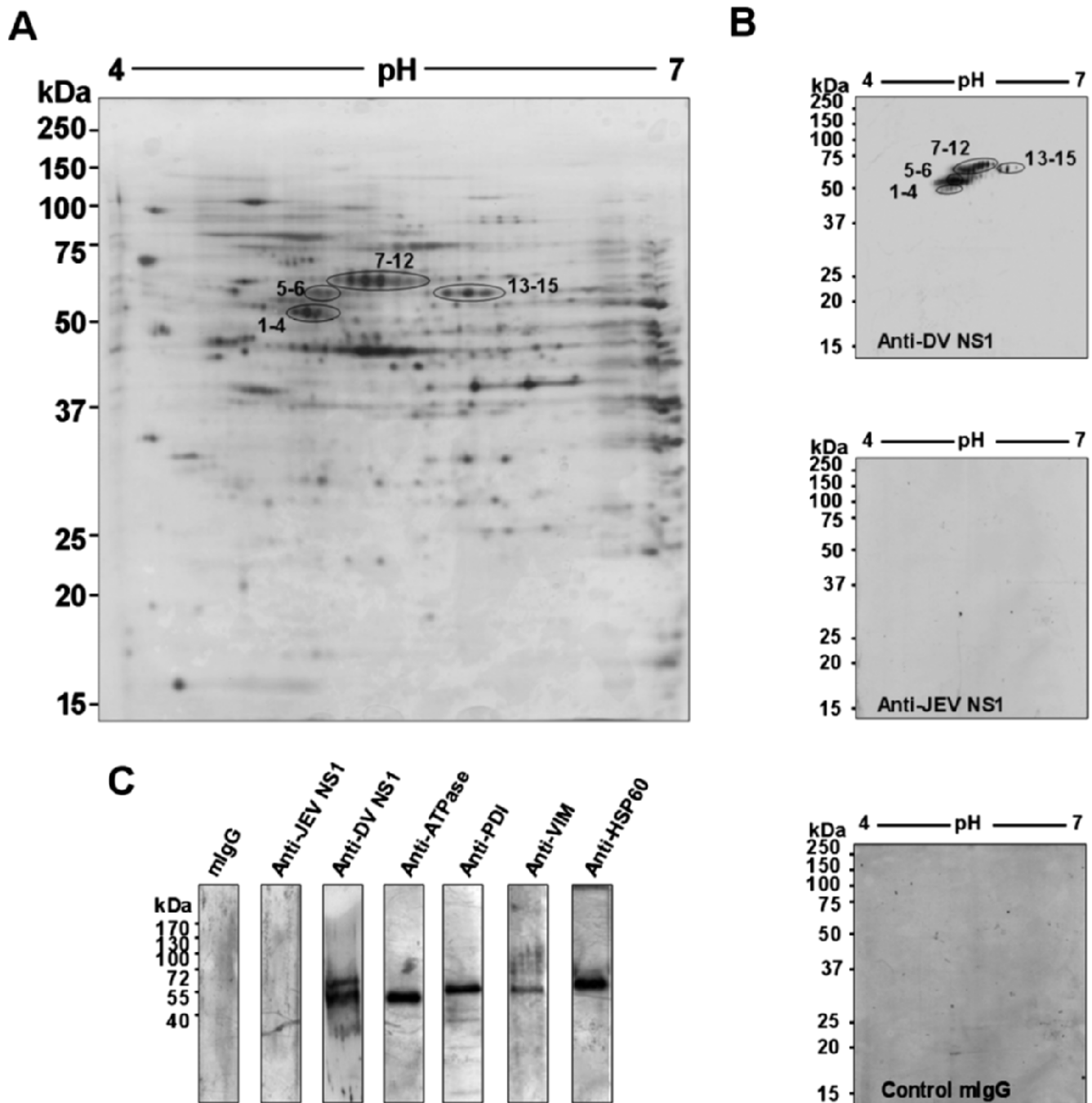


Figure 2. Identification of endothelial cell membrane proteins recognized by anti-DV NS1 antibodies. (A) 2-DE proteome map of HMEC-1 membrane proteins showing 15 protein spots chosen for identification. (B) 2-DE immunoblot profile of HMEC-1 membrane proteins with anti-DV NS1 IgG. (C) Antibodies against ATP synthase β chain (ATPase), protein disulfide isomerase (PDI), vimentin, and heat shock protein 60 (HSP60) react with biotin-labeled HMEC-1 membrane proteins in a Western blot analysis. Mouse IgG and anti-JEV NS1 IgG were negative controls.

an Emax microplate reader (Molecular Devices, Sunnyvale, CA) at 450 nm for TMB and 405 nm for ABTS.

Interactive complexes of target proteins and anti-DV NS1 were identified using a modified ELISA. The microtiter plates were coated with 1 μ g/well control mouse IgG, anti-JEV NS1, anti-DV NS1, anti-ATPase, anti-PDI, anti-vimentin, or anti-HSP60 at 37°C for 2 hrs. The wells were

blocked at 37°C for 2 hrs with 10% bovine serum albumin in PBS. After washing with 0.05% PBS-Tween 20, 2 μ g/well of endothelial cell membrane proteins were added and incubated at 37°C for 2 hrs. After washing with 0.05% PBS-Tween 20, anti-ATPase, anti-PDI, anti-vimentin, and anti-HSP60 antibodies, respectively, were then added in anti-NS1-coated wells at 4°C overnight, followed by HRP-

Table 1. HMEC-1 Membrane Proteins Recognized by Anti-DV NS1 Antibodies^a

SSP	Protein name	GenBank accession	Theoretical Mr/pl	Measured Mr/pl	Score ^b	M ^c	C ^d
1	ATP synthase β chain, mt precursor	P06576	56560/5.3	51249/5.0	0635	319	60.3
2	ATP synthase β chain, mt precursor	P06576	56560/5.3	50734/5.1	1426	374	70.7
3	Protein disulfide isomerase-related protein	NP005733	48121/5.0	50594/5.1	0305	134	30.5
4	Protein disulfide isomerase-related protein	NP005733	48121/5.0	50358/5.2	0278	082	18.6
5	Vimentin	XP042950	53685/5.1	58327/5.1	0463	124	26.6
6	Vimentin	XP042950	53685/5.1	58503/5.2	0668	071	15.2
7	Heat shock protein 60, mt precursor	P10809	61058/5.7	62316/5.2	0164	284	49.6
8	Heat shock protein 60, mt precursor	P10809	61058/5.7	62105/5.3	1239	259	45.2
9	Heat shock protein 60, mt precursor	P10809	61058/5.7	62890/5.4	2338	133	23.2
10	Heat shock protein 60, mt precursor	P10809	61058/5.7	62689/5.5	1463	309	53.9
11	Heat shock protein 60, mt precursor	P10809	61058/5.7	62780/5.5	0491	114	19.9
12	Heat shock protein 60, mt precursor	P10809	61058/5.7	62751/5.6	0282	076	13.3
13	Protein disulfide isomerase	CAA89996	56679/6.1	58465/5.8	0056	188	37.2
14	Protein disulfide isomerase	CAA89996	56679/6.1	58390/5.9	0632	233	46.1
15	Protein disulfide isomerase	CAA89996	56679/6.1	57912/6.0	0350	098	19.4

^a LC/MS/MS was used to identify the protein spots selected from 2-DE gel shown in Figure 1.

^b MOWSE, molecular weight search by MASCOT program.

^c The number of matched peptides (amino acids).

^d The percentage of sequence coverage (%).

conjugated anti-goat IgG or anti-rabbit IgG (1:4000 dilution). In target protein antibodies-coated wells, control mouse IgG, anti-JEV NS1 and anti-DV NS1, respectively, were added and incubated at 4°C overnight, followed by HRP-conjugated anti-mouse IgG (1:2000 dilution). After washing with 0.05% PBS-Tween 20 and incubation with TMB, the absorbance was measured using an Emax microplate reader (Molecular Devices) at 450 nm.

To determine the presence of anti-DV NS1 and anti-target protein antibodies in DHF patient sera, the microtiter plates were coated with 5 μ g/well DV NS1, PDI, vimentin, HSP60, or P311–330 for 2 hrs at 37°C, and then blocked with 10% bovine serum albumin in PBS for 2 hrs at 37°C. After washing with 0.05% PBS-Tween 20, 1 μ g/well IgG purified from DHF patient sera was added and incubated at 4°C overnight, followed by HRP-conjugated anti-human IgG (1:50,000 dilution) for 2 hrs at 37°C. After washing with 0.05% PBS-Tween 20 and incubation with TMB, the absorbance was measured using an Emax microplate reader at 450 nm.

Bioinformatic Analysis. Homologous peptide alignments were done using the LALIGN program (http://www.ch.embnet.org/software/LALIGN_form.html), a part of the FASTA package of sequence analysis programs. Prediction of potential antigenic regions in the protein sequences of DV NS1 and target proteins was done using the EMBOSS Antigenic program (<http://bioweb.pasteur.fr/seqanal/interfaces/antigenic.html>).

Results and Discussion

We previously proposed a mechanism of molecular mimicry in which antibodies against DV NS1 cross-react with endothelial cells and cause their activation and

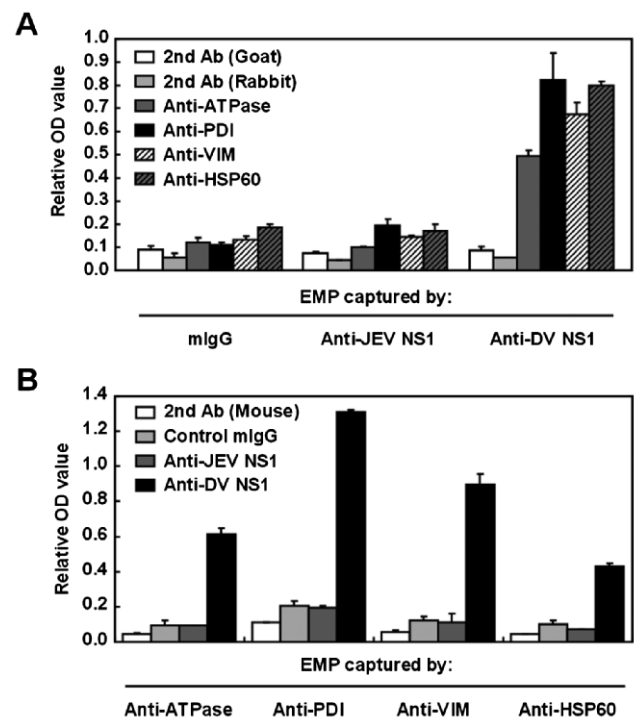


Figure 3. Binding of anti-DV NS1 antibodies to target proteins on endothelial cell membrane extracts. (A) Endothelial cell membrane extracts (EMP) were first captured by control mouse IgG, anti-JEV NS1, or anti-DV NS1 followed by detection using anti-ATP synthase β chain (ATPase), anti-protein disulfide isomerase (PDI), anti-vimentin, and anti-heat shock protein 60 (HSP60) antibodies and HRP-conjugated anti-goat IgG or anti-rabbit IgG secondary antibody (1:4000 dilution). (B) Endothelial cell membrane extracts (EMP) were first captured by anti-ATP synthase β chain (ATPase), anti-protein disulfide isomerase (PDI), anti-vimentin, and anti-heat shock protein 60 (HSP60) antibodies followed by detection using control mouse IgG, anti-JEV NS1, anti-DV NS1 antibodies and HRP-conjugated anti-mouse IgG secondary antibody (1:2000 dilution). Results are shown as mean $\pm$ SD from triplicate cultures.

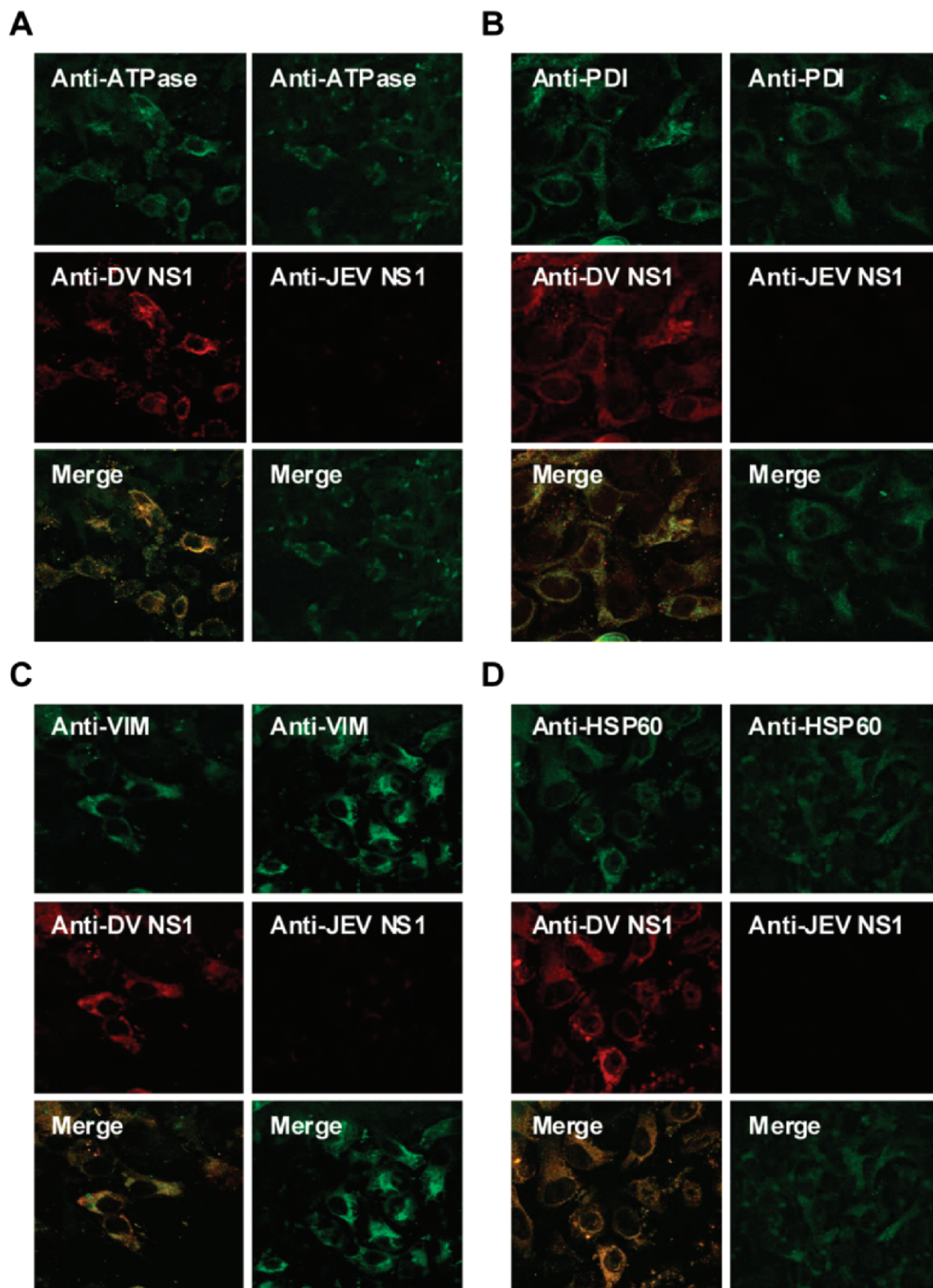


Figure 4. The binding sites of anti-DV NS1 co-localize with anti-ATP synthase β chain (ATPase), anti-protein disulfide isomerase (PDI), anti-vimentin, and anti-heat shock protein 60 (HSP60) antibodies. Nonfixed HMEC-1 cells were incubated with anti-DV NS1 IgG plus anti-ATPase (A), anti-PDI (B), anti-vimentin (C), or anti-HSP60 (D), and then with TRITC (red)- or FITC (green)-labeled secondary antibodies. The co-localizations of different antibodies shown in the merge (yellow) were detected using confocal microscopy. Anti-JEV NS1 IgG was used as a negative control.

apoptosis (25, 27). The endothelial binding activity of DV3-infected patient sera could be reduced by pretreatment with DV2 NS1 protein, indicating that the epitopes recognized by patient sera are shared between the endothelial cell surface molecules and NS1 of different DV serotypes (25). During the acute phase of disease, soluble NS1 could be detected in the plasma and its levels were correlated with viremia and disease severity (32–34). It has been reported that soluble NS1 can attach to some but not all endothelial cells. Selective vascular leakage in severe DV infection may be related to the relative ability of endothelial cells in different tissues to bind DV NS1 and to be targeted by anti-NS1 antibodies (35). We showed that the endothelial cell binding activity of anti-DV NS1 antibodies was enhanced when cells were pre-incubated with DV NS1 (Fig. 1). In addition, anti-DV NS1-mediated endothelial cell binding activity was also increased in the presence with DV. Heat inactivated DV2 (iDV2) showed a similar effect as that of live DV2. A combination of DV NS1 and DV2 or iDV2 caused an additive effect. We speculate that DV or iDV may trigger endothelial cells for surface expression of the molecules which are targeted by anti-DV NS1 antibodies. This proposed mechanism needs further investigation.

In present study, we identified several endothelial cell molecules recognized by anti-DV NS1. Membrane proteins from HMEC-1 cells were separated using 2-DE with isoelectric focusing (pH 4–7) (Fig. 2A) and then analyzed by Western blotting (Fig. 2B). After comparing the Western blots using anti-DV NS1 with anti-JEV NS1 or control mouse IgG (Fig. 2B), fifteen protein spots were selected for further analysis (Fig. 2A). We used liquid chromatography/tandem mass spectrometry to identify proteins, and then searched the NCBI and Swiss-Prot protein sequence databases. The target proteins recognized by anti-DV NS1 were the β chain of H^+ -transporter/ATP synthase (ATPase), protein disulfide isomerase (PDI) and PDI-related protein, vimentin intermediate filament, and heat shock protein 60 (HSP60) (Table 1).

The existence of these target proteins on the endothelial cell surface was confirmed using Biotin-labeled membrane extracts of HMEC-1 cells reactive with antibodies of the respective candidate proteins (Fig. 2C, lanes 4–7). The presence of these candidate target proteins on the endothelial surface has been previously reported (36–39). In addition, anti-DV NS1 antibodies bound to membrane proteins at molecular weights 55 to 60 kDa, corresponding to the sizes of these candidate proteins (Fig. 2C, lane 3).

Interactive complexes of anti-DV NS1 antibodies and target proteins were identified using a modified ELISA. The anti-DV NS1 antibodies coated on ELISA plate were allowed to bind with endothelial cell membrane proteins, and then the bound endothelial membrane proteins were detected using anti-ATPase, anti-PDI, anti-vimentin, and anti-HSP60 antibodies (Fig. 3A). Coating with mouse IgG and anti-JEV NS1 antibodies were used as negative controls. When anti-ATPase, anti-PDI, anti-vimentin, and

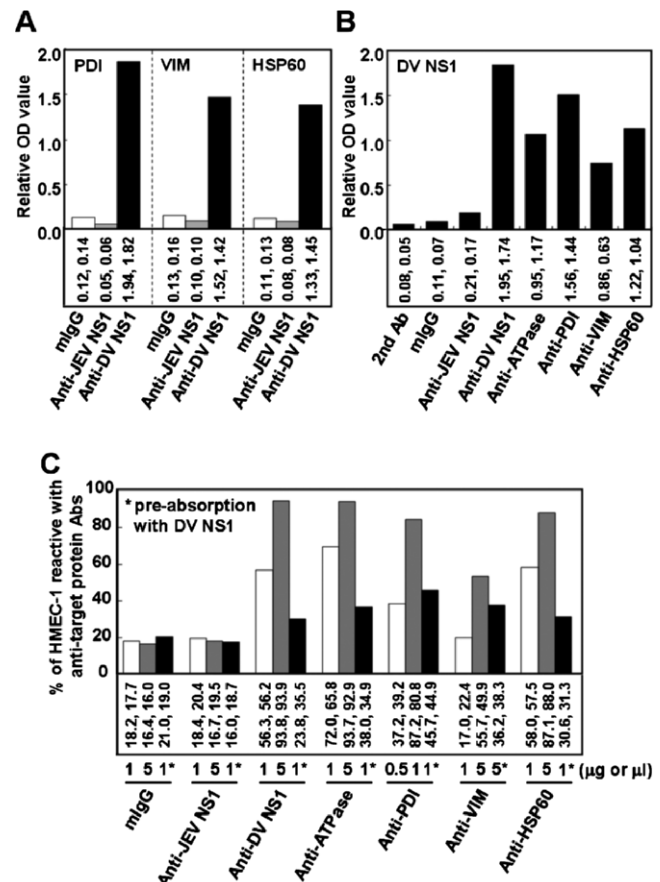
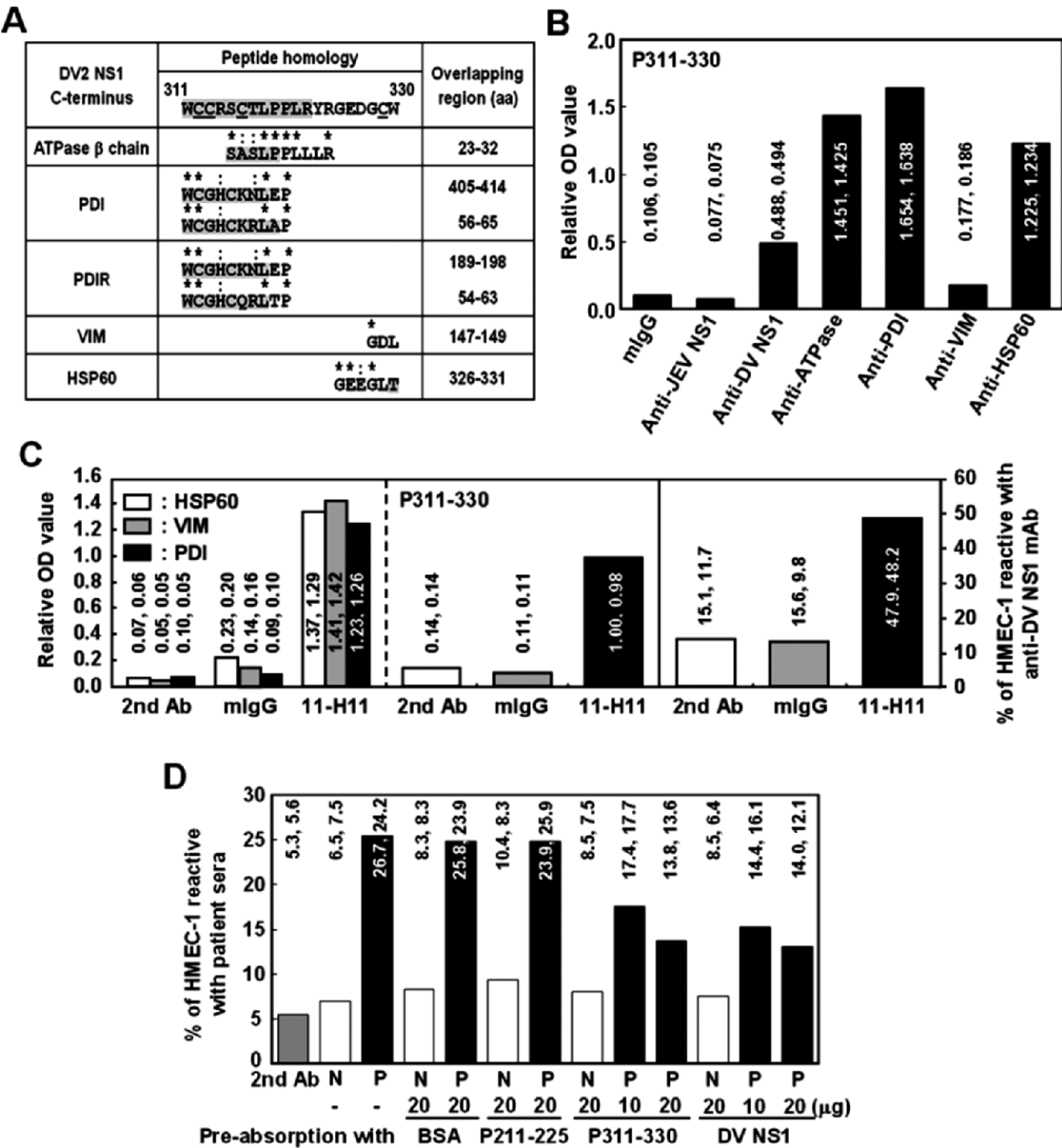


Figure 5. Binding activity of anti-DV NS1 and anti-target protein antibodies. (A) The cross-reactivity of anti-DV NS1 with protein disulfide isomerase (PDI), vimentin and heat shock protein 60 (HSP60) and (B) anti-target protein antibodies with DV NS1 protein were determined by ELISA. (C) The ability of anti-target protein antibodies to bind to endothelial cells decreased after pre-absorption with DV NS1. HMEC-1 cells were incubated with 1 or 5 μ g of anti-ATP synthase β chain (ATPase), anti-vimentin, or anti-HSP60, as well as 0.5 or 1 μ l of anti-PDI. Their binding ability was then analyzed using flow cytometry. In one set of experiments (*), 1 μ g of anti-ATPase or anti-HSP60, 5 μ g of anti-vimentin, and 1 μ l of anti-PDI were pre-absorbed with 1 μ g DV NS1 protein before being incubated with HMEC-1 cells. The numbers are the duplicate experimental values. Mouse IgG and anti-JEV NS1 IgG were negative controls.

anti-HSP60 were coated on ELISA plate to capture endothelial cell membrane proteins, anti-DV NS1 antibodies were then able to bind to these captured endothelial cell membrane proteins (Fig. 3B).

Using immunostaining of nonfixed HMEC-1 cells with anti-DV NS1 and anti-target protein antibodies followed by confocal microscopy, we found that these candidate autoantigens co-localized with the binding sites of anti-DV NS1 antibodies (Fig. 4). Anti-JEV NS1 showed no binding ability with endothelial cells. We next showed that anti-DV NS1 antibodies bound directly to the target proteins PDI, vimentin, and HSP60 (Fig. 5A). The ATPase was not tested because the purified protein is not commercially available. In addition, anti-target protein antibodies bound to the DV NS1 protein (Fig. 5B). We further found that the ability of the



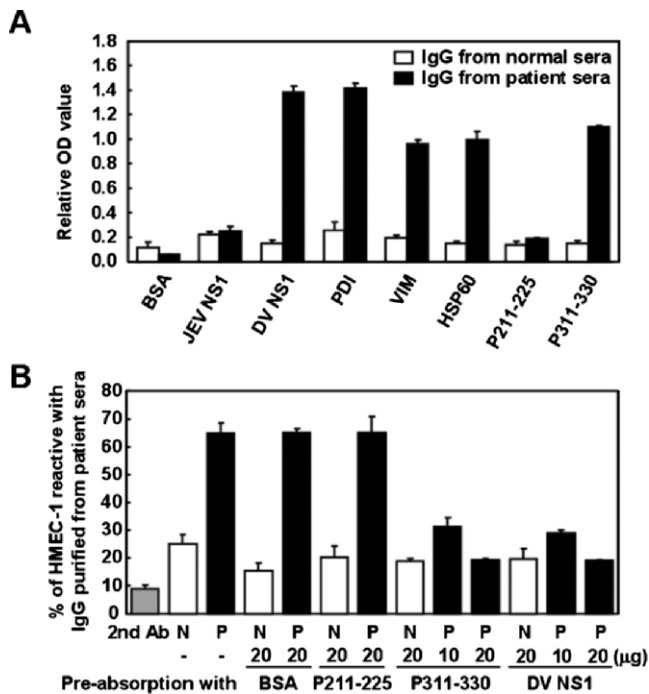


Figure 7. The binding activity of IgG purified from DHF patient sera to target proteins, P311–330, and endothelial cells. (A) The reactivity of purified IgG (1 μ g) with bovine serum albumin (BSA), JEV NS1, DV NS1, protein disulfide isomerase (PDI), vimentin, heat shock protein 60 (HSP60), P211–225, and P311–330 were determined by ELISA. (B) The binding ability of purified IgG to endothelial cells was reduced after pre-absorption with DV NS1 and P311–330. Purified IgG (5 μ g) from normal healthy control sera (N) or DHF mixed patient sera (P) were pre-absorbed with 10 or 20 μ g DV NS1, P311–330, P211–225, or BSA, and their binding ability to HMEC-1 cells was analyzed using flow cytometry.

anti-target protein antibodies to bind to HMEC-1 cells decreased after pre-absorption with DV NS1 (Fig. 5C).

We also observed the co-localization of these autoantigens with caveolin, a lipid raft marker (data not shown). Antibodies directed against ATPase inhibited endothelial cell surface-associated ATP synthesis and endothelial cell proliferation (40). It remains to be determined whether the cross-reactivity of anti-DV NS1 to the ATPase β chain has any effects related to our previous findings that anti-DV NS1 can induce endothelial cell apoptosis and immune activation (28, 29). Cell surface PDI reacts with nitric oxide (NO) and facilitates extracellular NO transfer to the intracellular compartment (41). In addition, PDI may act as a negative regulator of the NF- κ B pathway (42). The consequences of the binding of anti-DV NS1 to PDI on endothelial cells require further investigation. Vimentin intermediate filaments are important for maintaining vascular integrity. Vimentin has been identified as an endothelial cell autoantigen in some diseases (38). The production of autoantibodies against vimentin, however, may result from antigen spreading after the destruction of the endothelium. The 60-kDa family of heat shock proteins (HSPs) is highly conserved between humans and pathogens, which results in immunological cross-reactions. Autoantibodies acting

against HSP60 could induce endothelial cell death (43, 44). Whether anti-DV NS1 can trigger signaling pathways similar to those triggered by anti-HSP60 remains to be determined.

The cross-reactivity of anti-DV NS1 to target proteins and anti-target protein antibodies to DV NS1 indicates the existence of shared epitopes between DV NS1 and target proteins. Because of the disulfide bond arrangement of the DV NS1 protein, the cysteine-rich C-terminus of DV NS1 is thought to be where most of the antigenic conformations arise (45). The LALIGN alignment program annotated homologous peptide sequences between these candidate autoantigens and the DV NS1 protein C-terminal region (Fig. 6A). Moreover, using the EMBOSS program, we identified a potential region for antigenic determinants within amino acid residues 311–330 (P311–330) of DV NS1. We confirmed this result by showing the binding activity of anti-target protein antibodies to P311–330 (Fig. 6B). Anti-vimentin antibodies showed much less binding activity to P311–330, which suggested that it is not a major epitope shared between DV NS1 and vimentin. We also found that an anti-DV NS1 monoclonal antibody, 11-H11, showed cross-reactivity with HSP60, vimentin, and PDI. Furthermore, 11-H11 also showed binding with P311–330 and endothelial cells (Fig. 6C).

Identification of P311–330 as a predicted epitope was further confirmed using acute phase DHF patient sera. We found that DV NS1 and P311–330 pre-absorption reduced the binding of patient sera to HMEC-1 cells (Fig. 6D). Pre-absorption with bovine serum albumin or P211–225 did not have any inhibitory effect. Moreover, the IgG purified from DHF patient sera showed high binding activity with DV NS1, target proteins of PDI, VIM and HSP60, P311–330 (Fig. 7A), and also endothelial cells (Fig. 7B). Pre-absorption of IgG purified from DHF patient sera by DV NS1 or P311–330, but not BSA or P211–225, inhibited its binding activity to HMEC-1 cells (Fig. 7B). These results demonstrated that anti-DV NS1 present in the purified IgG of acute phase DHF patient sera could cross-react with endothelial cells and the potential epitope recognized by the purified antibodies was P311–330. Comparisons of the sequence of P311–330 between DV serotypes 3 and 2 show only one amino acid difference: a.a. 324 R $\rightarrow$ M. Between DV serotypes 1 and 2 there are two amino acid differences: a.a. 323 Y $\rightarrow$ F and a.a. 324 R $\rightarrow$ K. Finally, between DV serotypes 4 and 2 there are three amino acid differences: a.a. 318 L $\rightarrow$ M, a.a. 323 Y $\rightarrow$ F and a.a. 324 R $\rightarrow$ L. In addition, we previously reported no serotype specificity for anti-endothelial cell autoantibody production (46).

Autoimmune responses have been shown in a number of viral infections, such as human immunodeficiency virus (HIV), human hepatitis B virus (HBV), HCV, human cytomegalovirus (CMV), herpes simplex virus (HSV), Epstein-Barr virus (EBV), and severe acute respiratory syndrome-associated coronavirus (SARS-CoV) (47–53). Although autoimmune predominantly related to chronic virus infections, acute viral infections can induce transient

autoimmune responses, including generation of autoantibodies (53). In acute infection of DV, the anti-dengue NS1 antibody can cross-react with endothelial cells due to its molecular mimicry with ATPase, PDI, vimentin, and HSP60. The induction of autoimmune responses by viral infections suggests that such mechanisms may also apply to vaccination. The importance of our findings lies in the development of DV NS1 vaccines where cross-reactive epitopes may be eliminated to avoid autoimmune complications.

The use of recombinant proteins raises the question of whether such proteins represent native viral antigens. DV NS1 is expressed in mammalian cells in both membrane-associated and secreted forms, and may have a different molecular mass depending on the glycosylation state (54). Nevertheless, recombinant NS1 proteins or fragments that harbor similar immunological properties as native DV protein have been used successfully as diagnostic antigens (55). Our findings may lead to important new vaccine strategies by using an engineered DV NS1 protein lacking potentially harmful cross-reactive epitopes.

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