A Highly Conserved Region Between Amino Acids 221 and 266 of Dengue Virus Non-Structural Protein 1 Is a Major Epitope Region in Infected Patients

Magot Diata Omokoko, Sabar Pambudi, Supranee Phanthanawiboon, Promsin Masrinoul, Chayanee Setthapramote, Tadahiho Sasaki, Motoki Kuhara, Pongrama Ramasoota, Akifumi Yamashita, Itaru Hirai, Kazuyoshi Ikuta, and Takeshi Kurosu*

Department of Virology, Research Institute for Microbial Diseases (RIMD), Osaka University, Osaka, Japan; Center of Excellence for Antibody Research (CEAR), Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University, Ratchathewi, Bangkok, Thailand; Medical and Biological Laboratories Co., Ltd., Ina, Nagano, Japan; National Institute of Infectious Diseases, Tokyo, Japan; Laboratory of Microbiology, School of Health Sciences, Faculty of Medicine, University of the Ryukyus, Okinawa, Japan

Abstract. The immune response to dengue virus (DENV) infection generates high levels of antibodies (Abs) against the DENV non-structural protein 1 (NS1), particularly in cases of secondary infection. Therefore, anti-NS1 Abs may play a role in severe dengue infections, possibly by interacting (directly or indirectly) with host factors or regulating virus production. If it does play a role, NS1 may contain epitopes that mimic those epitopes of host molecules. Previous attempts to map immunogenic regions within DENV-NS1 were undertaken using mouse monoclonal Abs (MAbs). The aim of this study was to characterize the epitope regions of nine anti-NS1 human monoclonal Abs (HuMAbs) derived from six patients secondarily infected with DENV-2. These anti-NS1 HuMAbs were cross-reactive with DENV-1, -2, and -3 but not DENV-4. All HuMAbs bound a common epitope region located between amino acids 221 and 266 of NS1. This study is the first report to map a DENV-NS1 epitope region using anti-DENV MAbs derived from patients.

INTRODUCTION

Dengue is an arthropod-borne viral disease that has become a major public health concern in several countries, particularly tropical and subtropical areas of the world.¹ Dengue virus (DENV) has four serotypes (DENV-1, -2, -3, and -4)^{2,3} that cause dengue fever (DF). The disease can have mild or severe symptoms. For example, dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS) can be life-threatening. Humans can be serially infected by different DENV serotypes, and more severe cases are often seen in patients harboring secondary or serial infections.^{4,5}

DENV belongs to the genus *Flavivirus* within the family *Flaviviridae*, and it contains a positive-sense, single-stranded RNA genome. The viral genome is approximately 10,700 bases in length and encodes three structural proteins (capsid [C], pre-membrane [pre-M/M], and envelope [E]) and seven non-structural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). NS1 is a 46–55 kDa glycoprotein that comprises 352 amino acids. Unusual for a viral glycoprotein, it is released from infected mammalian cells and is not involved in virion formation.^{6–8} NS1 is either associated with intracellular membranes and the cell surface or secreted from DENV-infected cells as a soluble hexamer,⁶ which then circulates in the blood of infected patients.⁹ All *Flaviviruses* NS1 proteins have at least one *N*-linked glycosylation site, which is important for the processing and secretion of NS1.¹⁰ NS1 contains 12 conserved cystine residues, which form disulfide bonds between the following amino acids: 4–15, 55–143, 179–223, 280–329, 291–313, and 312–316. These disulfide bonds are responsible for the structural conformation adopted by NS1.¹¹ The dimerization of NS1 is dependent on the C-terminal region, which is also required for its secretion.¹²

NS1 acts as a soluble complement-fixing antigen in infected patients¹³; it is thought that secreted NS1 forms immune complexes that activate the complement system.^{14,15} High levels of circulating NS1 are detectable during the early stages of DENV infection and correlate with disease severity.^{16–19} Consequently, high levels of anti-NS1 antibodies (Abs) are produced, especially in secondary infection.²⁰ In addition, several studies show that mouse anti-NS1 monoclonal Abs (MAbs) cross-react with molecules expressed by platelets or endothelial cells; such cross-reactive antibodies may be involved in disease pathogenesis.^{14,21–25}

Here, we isolated nine anti-NS1 human MAbs (HuMAbs) obtained from patients harboring secondary DENV-2 infections at acute or convalescent stage²⁶ and characterized their reactivity against a series of recombinant NS1 proteins to identify the target epitopes on NS1.

MATERIALS AND METHODS

Production of HuMAbs. Anti-NS1 HuMAbs were prepared, and their reactivity was confirmed as described previously.²⁶ Briefly, a fusion partner cell line, SPYMEG, was previously established by fusion of the mouse myeloma cell line SP2/0-Ag14 (Riken Cell Bank RCB0209) and the human megakaryoblastic cell line MEG-01 (JCRB Cell Bank IFO050151) using polyethylene glycol 1500 (Roche Diagnostics, Mannheim, Germany). The fused cells were cultured in RPMI 1640 medium (NACALAI TESQUE, Kyoto, Japan) containing 10% fetal calf serum (FCS) in the presence of hypoxanthine-aminopterin-thymidine (HAT) for 5 days and further cultured in FCS-free RPMI medium for 3 days. The remaining cells were subsequently cultured with 10 µg/mL 8-azaguanine for 10 days, after which time limiting dilution was performed, generating the cell line designated SPYMEG. SPYMEG is a non-secretor of human or murine immunoglobulin (Ig), is 8-azaguanine-resistant, and is HAT-sensitive. Peripheral blood mononucleated cells (PBMCs) from secondarily infected DENV-2 patients were fused with SPYMEG cells at a ratio of 10:1 with polyethylene glycol 1500.

*Address of correspondence to Takeshi Kurosu, Department of Virology, Research Institute for Microbial Diseases, Osaka University, 3-1, Yamadaoka, Suita, Osaka 565-0871, Japan. E-mail: tkurosu@biken.osaka-u.ac.jp

Fused cells were cultured in Dulbecco's Modified Eagle medium (DMEM; Gibco, Gland Island, NY) supplemented with 15% FCS in 96-well microplates for 10–14 days in the presence of HAT. The first screening of the culture medium for antibodies specific to DENV was performed by focus-forming assay. Among these MAbs, anti-NS1-specific antibodies were selected by immunofluorescence assay (IFA) using 293T cells expressing DENV-2 NS1 antigen. Hybridomas were cultured in high-glucose DMEM containing 15% FCS supplemented with 1.5% BM-Condensed H1 (Roche Applied Science, Mannheim, Germany). This study was conducted according to the ethical principles for medical research involving human subjects at Mahidol University and approved by the institutional ethical review committees of Mahidol University. Written informed consent was obtained from each person at enrollment into the study.

HuMAb isotyping and subclass determination. To determine the subclasses of the MAbs, hybridomas culture supernatants were tested using a Human IgG Enzyme-Linked Immunosorbent Assay (ELISA) Quantitation Set (E80-104; Bethyl Laboratories, Inc., Montgomery, TX) in accordance with the manufacturer's instructions. IgG isotyping was performed by the polymerase chain reaction (PCR) method using messenger RNA (mRNA) from hybridomas. Total RNAs were isolated from hybridomas using Trizol reagent (Invitrogen, Carlsbad, CA) and reverse-transcribed with random primers. The resulting complementary DNA (cDNA) was used as a template to amplify each gene. The gene-specific primers used to amplify IgG1, -2, -3, and -4 are listed in Table 1. The same reverse (Rv) primer, Human IgG Rv, was used for IgG1, -2, -3, and -4. For IgG1 and IgG3, the same forward (Fw) primer, Human IgG1&3 Fw, was used. The amplified region contained the hinge region connecting Fab and Fc regions. By this set of primers, IgG1 generated a 211-bp band, whereas IgG3 generated a 346-bp band because of the insertion in the hinge region of IgG3. For IgG2 and IgG4, different forward primers, Human IgG2 Fw and Human IgG4 Fw, were used. IgG2 and IgG4 generated 207-bp and 210-bp bands, respectively. Collectively, the primer set Human IgG1&G3 Fw and Human IgG Rv was used to detect IgG1 and IgG3. The primer set Human IgG2 Fw and Human IgG Rv was used to detect IgG2. The primer set Human IgG4 Fw and Human IgG Rv was used to detect IgG4. The PCR mixture comprised 50 μ L reaction mixture containing 1 μ g cDNA, 10 mM KCl, 2 mM Tris-HCl (pH 8.0), 2.5 mM each deoxyribonucleotide triphosphate (dNTP), 10 μ M each primer, and 0.5 μ L ExTaq DNA Polymerase (TAKARA, Shiga, Japan). The PCR con-

ditions were as follows: 35 cycles of denaturation at 94.0°C for 30 seconds, annealing at 65.0°C for 30 seconds, and extension at 72.0°C for 30 seconds. The PCR products were subjected to agarose gel electrophoresis and visualized by staining with ethidium bromide. The sequences of amplified bands were confirmed by sequence analysis.

Viruses. DENV-1 (Mochizuki strain), DENV-2 (16681 strain), DENV-3 (H87 strain), DENV-4 (H241 strain), and Japanese encephalitis virus (JEV; JaGAR01 strain) were propagated in C6/36 cells at 28°C for 5–7 days. The culture media were stored at –80°C before use.

Plasmids construction. A plasmid expressing DENV-NS1 protein has been previously described²⁷; however, it was used as a PCR template to construct a mutant NS1 plasmid. DENV-2 NS1 cDNAs were amplified by PCR with different sets of primers. The PCR products were inserted into the *XhoI/BamHI* sites of pGEX-6P-1 (GE Healthcare, Buckinghamshire, United Kingdom) and transformed into *Escherichia coli* HB101 (Takara). All plasmids were verified by sequencing.

IFA. African green monkey kidney cells (Vero cells) were grown in Eagle's minimum essential medium (MEM; NACALAI TESQUE, Kyoto, Japan) supplemented with 10% FCS at 37°C in an atmosphere containing 5% CO₂. Vero cells were infected with DENVs or JEV (control cells were mock-infected) at a multiplicity of infection (MOI) of 0.1. Three days later, cells were fixed with 4% paraformaldehyde in phosphate-buffered solution (PBS) for 30 minutes, permeabilized with 1% Triton X-100 in PBS for 5 minutes, and then incubated with hybridoma culture medium for 1 hour. The cells were then washed three times with PBS and reacted with fluorescein isothiocyanate (FITC) -conjugated anti-human IgG antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 hour. After washing three times with PBS, cells were observed under a fluorescence microscope (Nikon Eclipse Ti, Inverted Microscope System; Nikon Instruments Inc., Melville, NY).

Sodium-dodecyl-sulfate polyacrylamide electrophoresis and immunoblot analysis. *E. coli* BL21-DE3-expressing recombinant NS1 proteins were lysed in sodium-dodecyl-sulfate polyacrylamide electrophoresis (SDS-PAGE) sample buffer in the absence of β -mercaptoethanol and heated at 100°C for 5 minutes. The proteins were separated on 12% SDS-PAGE gels and transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon; Millipore Corporation, Bedford, MA). The membranes were incubated with 5% (wt/vol) non-fat dry milk in 20 mM Tris-HCl (pH 8.0), 150 mM NaCl,

TABLE 1
Summary of patients' backgrounds and characterizations of anti-NS1 HuMAbs

Hybridomas clones	Patients	Diagnosis	Blood collection	Isotypes	Reactivity to infected cells (IFA)					Reactivity to DENV-2 mutants (WB)		
					DENV-1	DENV-2	DENV-3	DENV-4	JEV	S228T	E240Q	I242L
D23-5C7G1	D23	DF	Acute	IgG3	+	+	+	–	–	+	+	+
D23-2A8G5	D23	DF	Acute	IgG1	+	+	+	–	–	+	+/-	+
D25-2B11C3	D25	DF	Convalescent	IgG3	+	+	+/-	–	–	–	–	+
D25-4D3D2	D25	DF	Convalescent	IgG3	+	+	+	–	–	–	–	+
D25-4D4C3	D25	DF	Convalescent	IgG1	+	+	+	–	–	–	+/-	+
D26-5A2B12	D26	DF	Convalescent	IgG3	+	+	+	–	–	+/-	+	+
D27-1E8A4	D27	DHF	Convalescent	IgG1	+	+	+	–	–	+	+/-	+
D28-2B11F9	D28	DF	Convalescent	IgG*	+	+	–	–	–	+/-	+/-	+
D30-2B1G5	D30	DHF	Acute	IgG1	+	+	+	–	+	+	+/-	+

D23 and D26 were derived from the same patient at acute and convalescent phases, respectively. WB = western blotting; – = non-reactive; + = reactive; +/- = weakly reactive.
*Subclass of this IgG was not identified.

and 0.5% Tween 20 (PBS-T) and then incubated overnight with supernatants derived from the cultured hybridomas. After washing with PBS-T, the membranes were incubated with the horseradish peroxidase (HRP) -conjugated anti-human IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 hour. Alternatively, the membrane was first incubated with anti-glutathione S-transferase (GST) Abs (Sigma, St. Louis, MO) followed by HRP-conjugated anti-rabbit Abs (Jackson ImmunoResearch Laboratories). Reactive NS1 proteins were visualized using the ECL Western blotting detection reagent (GE Healthcare, Freiburg, Germany).

Analysis of flaviviruses sequences and amino acids variations of epitope region recognized by the MAbs. In total, 1,392 DENV-1 sequences, 1,094 DENV-2 sequences, 707 DENV-3 sequences, and 111 DENV-4 sequences were collected from the National Center of Biotechnology Information (NCBI) protein database. The amino acids sequences within the epitope region were analyzed using Bioedit version 7.0.9.0 (Ibis Biosciences; Abbott, Carlsbad, CA). Amino acid variations were displayed on an Entropy H(x) plot.

Site-directed mutagenesis of key residues within the amino acids region 221–266. Single amino acid substitutions were introduced into pGEX-6P-1.NS1.221-352 by PCR. A series of complementary sense and antisense oligonucleotide primers was synthesized to amplify pGEX-6P-1.NS1.221-352 by PCR. The PCR products were digested with *DpnI* and transformed into *E. coli* HB101. Nucleotide sequence analysis of the pGEX-6P-1.NS1.221-352 mutants was performed using a BigDye Terminator v3.1 Cycle Sequencing Kit with an ABI PRISM 3130XL genetic analyzer (Applied Biosystems, Foster City, CA) and the following set of primers: 5'-GGGCTGGCAAGC CACGTTTGGTG-3' and 5'-CCGGGAGCTGCATGTGTC AGAGG-3'. Data were analyzed using SeqScape v2.5 software (Applied Biosystems).

RESULTS

Characterization of anti-NS1 HuMAbs. Nine anti-NS1 HuMAbs were obtained from patients harboring secondary DENV-2 infections: two patients were in the acute phase (D23-5C7G1/D23-2A8G5 and D30-2B1G5) and four patients were in the early convalescent phase (D25-2B11C3/D25-4D3D2/D25-4D4C3, D26-5A2B12, D27-1E8A4, and D28-2B11F9). The reactivity of these MAbs against NS1 was previously confirmed.²⁶ We next examined the serological reactivity of the HuMAbs against all four DENV serotypes plus JEV using IFA. Anti-E HuMAb D23-1G7C2²⁶ was used as a positive control to confirm that Vero cells had been infected with each virus (Figure 1). All HuMAbs were cross-reactive to DENV-1 and DENV-2. Most of the HuMAbs weakly reacted with DENV-3, although D25-2B11C3 and D25-4D3D2 very weakly reacted with DENV-3 and D28-2B11F9 did not react with DENV-3. Interestingly, none of HuMAbs reacted to DENV-4. Only D25-4D3D2 reacted with JEV and DENV-1, -2, and -3.

Subclass analysis of the anti-NS1 HuMAbs. ELISAs showed that all of the HuMAbs belonged to the IgG class (Figure 2A). We next examined the IgG subclass. The subclass is physiologically important, because it determines, for example, whether an antibody activates complement or is involved in opsonization.²⁸ The cDNAs derived from hybridomas were PCR-amplified to detect the IgG subclasses. Two subclasses, IgG1 and IgG3, were predominant. Hybrid-

FIGURE 1. Serotype specificity of anti-NS1 HuMAbs. Vero cells were infected with DENV-1, DENV-2, DENV-3, DENV-4, or JEV. The reactivity of each of the MAbs with cells infected with each virus was then examined. The anti-E HuMAb, D23-1G7C2, was used as a positive control.

omas D25-4D4C3, D27-1E8A4, D23-5C7G1, and D30-2B1G5 produced IgG1 (211 bp), whereas D25-2B11C3, D25-4D3D2, D26-5A2B12, and D23-2A8G5 produced IgG3 (346 bp) antibodies (Figure 2B).

Anti-NS1 HuMAbs bind a highly conserved epitope region within amino acids region 221–266. We next examined the epitope region recognized by these MAbs using a series of truncated DENV-2 NS1 mutants (Figure 3A). Each construct contains a region (full-length [1–352 amino acids], 267–352 amino acids, 221–352 amino acids, 197–352 amino acids, 169–352 amino acids, or 141–352 amino acids of NS1). First, we used anti-GST antibody to confirm equal expression of all truncated proteins (Figure 3B). All HuMAbs (except for GST-NS1.267-352) reacted with all of the truncated NS1 mutants, suggesting that they all recognized the region between 221 and 266 amino acids of the DENV-2 NS1 protein. Second, we performed a more detailed analysis by comparing the amino acids sequence corresponding to the epitope regions 221–266 amino acids within each of the DENV serotypes and within JEV. We found that the region located between 225 and 245 amino acids was highly conserved in DENV-1, -2, and -3 but not in DENV-4 or JEV (Figure 4). We suspected that this region contained an epitope recognized by the MAbs, because most of the HuMAbs reacted with DENV-1, -2, and -3 but not DENV-4 (Figure 1). Among the

FIGURE 2. Isotyping and IgG subclass examination. (A) Hybridoma culture supernatants were tested by ELISA to determine the isotypes of the HuMAbs. Each ELISA included three positive controls (Ctrl; Ctrl IgA, Ctrl IgG, and Ctrl IgM). (B) IgG subclasses were determined by reverse transcription PCR. cDNA was reverse-transcribed from RNA isolated from each hybridoma and amplified using three sets of human IgG primers. The expected sizes of the PCR products were as follows: 211 bp for IgG1, 207 bp for IgG2, 346 bp for IgG3, and 210 bp for IgG4. Arrows to the right show the position of each IgG subclass.

conserved amino acids within this region, T228, Q240, and L242 were unique to DENV-4 (Figure 4).

Mutagenesis of the amino acids within amino acids region 221–266. We next used a pGEX-6P-1.NS1.221-352 mutant to examine differences in reactivity to DENV-1, -2, and -3 and DENV-4 (Figure 1). Each mutant harbored a single mutation, S228T, E240Q, or I242L. D25-2B11C3, D25-4D3D2, and D28-2B11F9 reacted with the I242L mutant NS1 protein and DENV-2 wild-type (WT) NS1 but not the S228T and E240Q mutant NS1 proteins (Figure 5). D26-5A2B12 did not react with the S228T mutant. D27-1E8A4, D30-2B1G5, and D23-2A8G5 reacted with the S228T and I242L mutant NS1 proteins but not with the E240Q mutant. Only D23-5C7G1 reacted with all mutant NS1 proteins. These results suggest that the region containing S228 and E240 is a common epitope for most of HuMAbs, because eight of nine HuMAbs did not react with the S228T or E240Q mutants. D23-5C7G1 reacted with both S228T and E240Q mutant proteins. However, we cannot exclude the possibility that the epitope region recognized by D23-5C7G1 is similar to that recognized by the other HuMAbs, because the region between S228 and E240 contains 12 amino acids, suggesting that this region is probably long enough to be recognized as an epitope region by MAbs. Otherwise, taken together, these observations indicate that the region containing amino acids 228S and 240E is a major epitope recognized by the anti-DENV HuMAbs.

The NS1 region containing amino acids 225–245 is highly conserved in each DENV serotype. To confirm the conservation of the epitope region, the amino acid sequences of the NS1 proteins were analyzed using 3,304 reference sequences for DENV-1, -2, -3, and -4 obtained from the NCBI database (Figure 6). A highly conserved region was identified at amino acids 225–245 in each serotype. A corresponding region at

amino acids 226–244 (except amino acid 240) was highly conserved in the DENV-1, -2, -3, and -4 NS1 proteins examined in the present study. The high entropy plot for amino acid 240 arose, because DENV-4 contains a conserved Q residue (Figure 6D). Thus, the region at amino acids 225–245 seems to be a common epitope region for DENV-1, -2, and -3.

DISCUSSION

The HuMAbs derived from patients with secondary DENV-2 infection cross-reacted with other serotypes of NS1 (although not with DENV-4 NS1) and belonged to the IgG1 and IgG3 subclasses. Unexpectedly, all of the HuMAbs (except D23-5C7G1) bound to amino acid region 225–245 within NS1. This result is likely to be a common epitope region within DENV-1, -2, and -3 but not within DENV-4. This epitope region has not been reported previously, which may be because of the different origins of the MAbs used in previous studies.^{10,29–37} Here, we performed epitope mapping using HuMAbs derived from DENV-infected patients, whereas previous studies used mouse MAbs. According to the results of the previous studies, almost the entire NS1 region could act as an epitope region recognized by mouse MAbs, whereas HuMAbs map a limited region located between amino acids 221 and 266. However, there is a limitation in our study, because MAbs used in this study were generated from patients secondarily infected with DENV-2.²⁶ Figure 7B compares the reported NS1 epitopes regions.^{10,29–39} These studies hypothesized that anti-NS1 antibodies cross-react with host molecules and subsequently, induce plasma leakage. Some mouse anti-NS1 MAbs cross-react with host molecules that share similar epitopes with NS1.^{16,38–41} If we clarify whether anti-NS1 Abs are involved in disease pathogenesis by cross-reacting with host molecules, studies should also be performed using Abs derived from

FIGURE 3. Epitope mapping of anti-NS1 HuMAbs. (A) A series of GST-NS1 fusion proteins was constructed. (B) Lysates of *E. coli* BL21DE3 expressing GST fusion proteins were subjected to SDS-PAGE, and the proteins were transferred to PVDF membranes. The membranes were then stained with an anti-GST Ab or the following HuMAbs: (C) D25-2B11C3, (D) D25-4D3D2, (E) D25-4D4C3, (F) D26-5A2B12, (G) D27-1E8A4, (H) D28-2B11F9, (I) D30-2B1G5, (J) D23-2A8G5, and (K) D23-5C7G1.

patients rather than mouse-derived Abs. Although we performed a basic local alignment search tool (BLAST) search as part of the present study, we could not identify any host molecular sequences that were homologous to this epitope

region within NS1 (data not shown). Additional studies using HuMAbs are needed to understand the mechanisms underlying the cross-reactivity between anti-NS1 antibodies and host molecules.

Flavivirus strains	Peptide sequences											
	221	230	240	250	260	266						
DENV-1 Mochizuki	KTCV	WPKSHTLWSNGVLESE	MTIPKLYGGPISQHNYPGYSTQTAG									
DENV-2 16681	.N.H			NLA..V		H..IT						
DENV-3 H87	...T		D.....	SLA.....	H.....	H.....						
DENV-4 H241	...I	...T		Q..L	A..A..F		Q..A..V					
JEV JaGAr01	.S.T	ET	GD..E	L	HTIA..K.K..R.E..K..NQ							
		228	240 242									

FIGURE 4. Amino acid sequence alignment of epitope amino acid region 221–266 within DENV-1, DENV-2, DENV-3, DENV-4, and JEV. The dotted rectangle indicates a highly conserved region (amino acids 225–245) within DENV-1, -2, -3, and -4. The rectangles indicate amino acids unique to DENV-4 (amino acids 228, 240, and 242).

FIGURE 5. Reactivity of anti-NS1 HuMAbs with the S228T, E240Q, and I242L mutant NS1 proteins. Lysates of *E. coli* BL21DE3 expressing GST-NS1 (amino acids 221–352) mutants were subjected to SDS-PAGE, and the proteins were transferred to PVDF membranes. The membranes were then stained with (A) anti-GST Ab, (B) D25-2B11C3, (C) D25-4D3D2, (D) D25-each, (E) D26-5A2B12, (F) D27-1E8A4, (G) D28-2B11F9, (H) D30-2B1G5, (I) D23-2A8G5, and (J) D23-5C7G1.

FIGURE 6. Entropy plot showing the amino acid variations within the epitope region amino acids 221–266 within DENV-1, -2, -3, and -4. Values are derived from the sequences of (A) DENV-1, (B) DENV-2, (C) DENV-3, (D) DENV-4, and (E) all DENV serotypes. The rectangles indicate the highly conserved region (amino acids 225–245) within DENV-1, -2, -3, and -4. The amino acids unique to each serotype are shown in bold. The two amino acids indicated by the X (amino acids 240 and 242) are variable.

We used site-directed mutagenesis and entropy plots to predict that the region containing amino acids 228–240 would be a major common epitope region for DENV-1, -2, and -3 (Figures 5 and 6). Interestingly, another study used a combination of the enzyme-linked immunosorbent spot (ELISPOT) and *in silico* analyses in human leukocyte antigen (HLA) transgenic mice to identify a similar region (amino acids 229–239) as a T-cell epitope.⁴² Previous studies used proteolytic digestion experiments

to show that *Flavivirus* NS1 comprises three domains (Ds; DI, DII, and DIII).^{34,43,44} We found that the major epitope region, containing amino acids 228–240, is located at the junction between DII and DIII (Figure 7A and C).^{34,43,44} More recently, the crystal structure of *Flavivirus* NS1 was unraveled,⁴⁵ showing that the region of amino acids 228–240 was located in the long, extended loop structure, called spaghetti loop, spanning from amino acids 219 to 272, and this region was exposed to

FIGURE 7. Summary of the epitope regions within DENV-NS1 proteins. (A) Linear representation of the NS1 protein. Three domains (DI, DII, and DIII) are expressed. The white circles and dashed lines indicate 12 conserved cysteine residues and their disulfide linkages, respectively. Glycosylation sites at amino acids 130 and 207 are represented by stars. (B) Previously reported B-cell epitope regions are indicated by grey rectangles, and the newly identified epitope region (amino acids 221–266) is indicated by the black rectangle. Numbers in parentheses indicate the relevant literature references. (C) Schematic representation of the three domains of the NS1 protein. Proposed tertiary arrangement of the three structural domains within an NS1 monomer based on overlapping epitopes recognized by MAbs. HuMAbs target the junction between DII and DIII (indicated by the bracket). The numbers indicate amino acid positions. The domain structure is derived from the work by Muller and Young.⁴⁴

the surface of NS1 molecule. These observations suggest that this epitope region is exposed on the surface of the NS1 molecule and imply that this region may be a suitable B-cell epitope. The region containing amino acids 228–240 is likely to be a common epitope within DENV-1, -2, and -3.

The HuMAbs used in the present study belonged to the IgG1 or IgG3 subclasses (at a ratio of 50:50) (Figure 2B). Normally, the relative concentrations of IgG1, IgG2, IgG3, and IgG4 in healthy humans are 70–80%, 13–18%, 6–8%, and 3%, respectively.⁴⁶ Both IgG1 and IgG3 activate complement

through the classical pathway.²⁸ Strong complement activation is often observed in DENV-infected patients and may be related to disease severity.^{47,48} Anti-NS1 Abs are likely to be, at least in part, involved in complement activation.

We identified the epitope region recognized by anti-DENV Abs using a series of N-terminally truncated mutant NS1 proteins (Figure 4). We also used a series of C-terminally truncated mutant NS1 proteins; however, the HuMAbs did not recognize NS1 proteins lacking the C terminus. Moreover, the HuMAbs showed a marked reduction in their ability to recognize NS1 proteins run in reducing SDS-PAGE gels (data not shown). These observations indicate that anti-NS1 Abs recognized the epitope region when it was bent by disulfide bonds located in C-terminal region of NS1. In other words, the epitope region between amino acids 221 and 266 is not a completely linear epitope, although it was reactive on Western blots (Figure 4). Thus, internal disulfide bonds located in the C-terminal region of NS1 are likely to be important for maintaining the structure of the epitope.

At first, we expected that the anti-NS1 HuMAbs would cross-react with virus particles, because mouse anti-NS1 MAbs recognized the prM protein and showed neutralizing and antibody-dependent enhancement (ADE) activities against DENV⁴⁹; however, none of the anti-NS1 HuMAbs showed neutralizing or ADE activity (data not shown). Because we examined only nine HuMAbs, we cannot confirm whether patient-derived anti-NS1 Abs cross-react with virus particles; however, the physiological relevance of anti-NS1 Abs remains unclear.

Taken together, the results of the present study suggest that the NS1 epitope region recognized by Abs derived from dengue patients is different from the region recognized by murine MAbs. These findings may provide new insights into the pathogenic role played by anti-NS1 Abs.

Received October 26, 2013. Accepted for publication February 16, 2014.

Published online April 28, 2014.

Acknowledgments: The authors thank BioEdit (Stockport, United Kingdom) for English proofreading.

Financial support: This work was supported by Grant-in-Aid for Scientific Research (C) 21790444 from the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) and the Japan Science and Technology Agency (JST)/Japan International Cooperation Agency (JICA) as part of Science and Technology Research Partnership for Sustainable Development (SATREPS) Grant 08080924.

Authors' addresses: Magot Diata Omokoko, Sabar Pambudi, Supranee Phanthanawiboon, Tadahiro Sasaki, Kazuyoshi Ikuta, and Takeshi Kurosu, Department of Virology, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan, E-mails: omokoko@biken.osaka-u.ac.jp, sabarp@biken.osaka-u.ac.jp, supraneeoui@biken.osaka-u.ac.jp, sasatada@biken.osaka-u.ac.jp, ikuta@biken.osaka-u.ac.jp, and tkurosu@biken.osaka-u.ac.jp. Promsin Masrinoul, Institute of Molecular Biosciences, Mahidol University, Salaya, Nakhon Pathom, Thailand, E-mail: promsin.mas@mahidol.ac.th. Chayanee Sethapramote and Pongrama Ramasoota, Center of Excellence for Antibody Research (CEAR), Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University, Ratchathewi, Bangkok, Thailand, E-mails: lookmoo_cu@hotmail.com and pongrama.ram@mahidol.ac.th. Motoki Kuhara, Medical and Biological Laboratories Co. Ltd., Nagano, Japan, E-mail: kuhara.motoki@mbi.co.jp. Akifumi Yamashita, National Institute of Infectious Diseases, Tokyo, Japan, E-mail: uhmin@niid.go.jp. Itaru Hirai, Laboratory of Microbiology, School of Health Sciences, Faculty of Medicine, University of the Ryukyus, Okinawa, Japan, E-mail: hirai@med.u-ryukyuu.ac.jp.

Reprint requests: Takeshi Kurosu, Department of Virology, Research Institute for Microbial Diseases, Osaka University, 3-1, Yamadaoka, Suita, Osaka 565-0871, Japan, E-mail: tkurosu@biken.osaka-u.ac.jp.

REFERENCES

- Gubler DJ, 2002. The global emergence/resurgence of arboviral diseases as public health problems. *Arch Med Res* 33: 330–342.
- Calisher CH, Karabatsos N, Dalrymple JM, Shope RE, Porterfield JS, Westaway EG, Brandt WE, 1989. Antigenic relationships between flaviviruses as determined by cross-neutralization tests with polyclonal antisera. *J Gen Virol* 70: 37–43.
- Chambers T, Hahn C, Galler R, Rice C, 1990. Flavivirus: genome organization, expression, and replication. *Annu Rev Microbiol* 44: 649–688.
- Halstead S, Nimmannitya S, Cohen S, 1970. Observations related to pathogenesis of dengue hemorrhagic fever. IV. Relation of disease severity to antibody response and virus recovered. *Yale J Biol Med* 42: 311–328.
- Kouri GP, Guzmán MG, Bravo JR, Triana C, 1981. Dengue haemorrhagic fever/dengue shock syndrome: lessons from the Cuban epidemic. *Bull World Health Organ* 67: 375–380.
- Flamand M, Megret F, Mathieu M, Lepault J, Rey FA, Deubel V, 1999. Dengue virus type 1 nonstructural glycoprotein NS1 is secreted from mammalian cells as a soluble hexamer in a glycosylation-dependent fashion. *J Virol* 73: 6104–6110.
- Noisakran S, Dechawawat T, Rinkawek P, 2007. Characterization of dengue virus NS1 stably expressed in 293T cell lines. *J Virol Methods* 142: 67–80.
- Pryor MJ, Wright PJ, 1994. Glycosylation mutants of dengue virus NS1 protein. *J Gen Virol* 75: 1183–1187.
- Gutsche I, Coulibaly F, Voss JE, Salmon J, D'Alayer J, Ermonval M, Larquet E, Charneau P, Krey T, Mégret F, Guittet E, Rey FA, Flamand M, 2011. Secreted dengue virus nonstructural protein NS1 is an atypical barrel-shaped high-density lipoprotein. *Proc Natl Acad Sci USA* 108: 8003–8008.
- Mason PW, Zfigel U, Semproni AR, Fournier MJ, Mason TL, 1990. The antigenic structure of dengue type 1 virus envelope and NS1 proteins expressed in *Escherichia coli*. *J Gen Virol* 71: 2107–2114.
- Wallis TP, Huang C-Y, Nimkar SB, Young PR, Gorman JJ, 2004. Determination of the disulfide bond arrangement of dengue virus NS1 protein. *J Biol Chem* 279: 20729–20741.
- Pryor MJ, Wright PJ, 1993. The effects of site-directed mutagenesis on the dimerization and secretion of the NS1 protein specified by dengue virus. *Virology* 194: 769–780.
- Russell P, Chiewsilp D, Brandt WE, 1970. Immunoprecipitation analysis of soluble complement-fixing antigens of dengue viruses. *J Immunol* 105: 838–845.
- Avirutnan P, Punyadee N, Noisakran S, Komoltri C, Thiemmecca S, Auethavornanan K, Jairungsri A, Kanlaya R, Tangthawornchaikul N, Puttikhunt C, Pattanakitsakul S-N, Yenchitsomanus P-T, Mongkolsapaya J, Kasinrerk W, Sittisombut N, Husmann M, Blettner M, Vasanawathana S, Bhakdi S, Malasit P, 2006. Vascular leakage in severe dengue virus infections: a potential role for the nonstructural viral protein NS1 and complement. *J Infect Dis* 193: 1078–1088.
- Green S, Rothman A, 2006. Immunopathological mechanisms in dengue and dengue hemorrhagic fever. *Curr Opin Infect Dis* 19: 429–436.
- Alcon S, Talarmin A, Debruyne M, Falconar AKI, Deubel V, Flamand M, 2002. Enzyme-linked immunosorbent assay specific to dengue virus type 1 nonstructural protein NS1 Reveals circulation of the antigen in the blood during the acute phase of disease in patients experiencing primary or secondary infections. *J Clin Microbiol* 40: 376–381.
- Hu D, Di B, Ding X, Wang Y, Chen Y, Pan Y, Wen K, Wang M, Che X, 2011. Kinetics of non-structural protein 1, IgM and IgG antibodies in dengue type 1 primary infection. *Virol J* 8: 47.
- Libraty DH, Young PR, Pickering D, Endy TP, Kalayanaroj S, Green S, Vaughn DW, Nisalak A, Ennis FA, Rothman AL, 2002. High circulating levels of the dengue virus nonstructural protein NS1 early in dengue illness correlate with the development of dengue hemorrhagic fever. *J Infect Dis* 186: 1165–1168.
- Young PR, Hilditch PA, Bletchly C, Halloran W, 2000. An antigen capture enzyme-linked immunosorbent assay reveals high levels of the dengue virus protein NS1 in the sera of infected patients. *J Clin Microbiol* 38: 1053–1057.

20. Valdés K, Alvarez M, Pupo M, Vázquez S, Rodríguez R, Guzmán MG, 2000. Human dengue antibodies against structural and nonstructural proteins. *Clin Diagn Lab Immunol* 7: 856–857.
21. Avirutnan P, Fuchs A, Hauhart RE, Somnuk P, Youn S, Diamond MS, Atkinson JP, 2010. Antagonism of the complement component C4 by flavivirus nonstructural protein NS1. *J Exp Med* 207: 793–806.
22. Avirutnan P, Hauhart RE, Somnuk P, Blom M, Diamond MS, Atkinson JP, 2011. Binding of flavivirus nonstructural protein NS1 to C4b binding protein modulates complement activation. *J Immunol* 187: 424–433.
23. Cheng H-J, Lei H-Y, Lin C-F, Luo Y-H, Wan S-W, Liu H-S, Yeh T-M, Lin Y-S, 2009. Anti-dengue virus nonstructural protein 1 antibodies recognize protein disulfide isomerase on platelets and inhibit platelet aggregation. *Mol Immunol* 47: 398–406.
24. Lin C-F, Lei H-Y, Shiao A-L, Liu C-C, Liu H-S, Yeh T-M, Chen S-H, Lin Y-S, 2003. Antibodies from dengue patient sera cross-react with endothelial cells and induce damage. *J Med Virol* 69: 82–90.
25. Liu I-J, Chiu C-Y, Chen Y-C, Wu H-C, 2011. Molecular mimicry of human endothelial cell antigen by autoantibodies to nonstructural protein 1 of dengue virus. *J Biol Chem* 286: 9726–9736.
26. Setthapramote C, Sasaki T, Puiprom O, Limkittikul K, Pitaksajakul P, Pipattanaboon C, Sasayama M, Leuangwutiwong P, Phumratanaprapin W, Channachanan S, Kusolsuk T, Jittmittraphap A, Asai A, Arias JF, Hirai I, Kuhara M, Okuno Y, Kurosu T, Ramasoota P, Ikuta K, 2012. Human monoclonal antibodies to neutralize all dengue virus serotypes using lymphocytes from patients at acute phase of the secondary infection. *Biochem Biophys Res Commun* 423: 867–872.
27. Kurosu T, Chaichana P, Yamate M, Anantapreecha S, Ikuta K, 2007. Secreted complement regulatory protein clusterin interacts with dengue virus nonstructural protein 1. *Biochem Biophys Res Commun* 362: 1051–1056.
28. Jefferis R, Lund J, 2002. Interaction sites on human IgG-Fc for FcγR: current models. *Immunol Lett* 82: 57–65.
29. Chen Y, Pan Y, Guo Y, Qiu L, Ding X, Che X, 2010. Comprehensive mapping of immunodominant and conserved serotype- and group-specific B-cell epitopes of nonstructural protein 1 from dengue virus type 1. *Virology* 398: 290–298.
30. Falconar AKI, 2008. Monoclonal antibodies that bind to common epitopes on the dengue virus type 2 nonstructural-1 and envelope glycoproteins display weak neutralizing activity and differentiated responses to virulent strains: implications for pathogenesis and vaccines. *Clin Vaccine Immunol* 15: 549–561.
31. Falconar AKI, Young PR, Miles MA, 1994. Precise location of sequential dengue virus subcomplex and complex B cell epitopes on the nonstructural-1 glycoprotein. *Arch Virol* 137: 315–326.
32. Jiang L, Zhou J-M, Yin Y, Fang D-Y, Tang Y-X, Jiang L-F, 2010. Selection and identification of B-cell epitope on NS1 protein of dengue virus type 2. *Virus Res* 150: 49–55.
33. Masrinoul P, Diata MO, Pambudi S, Limkittikul K, Ikuta K, Kurosu T, 2011. Highly conserved region 141–168 of the NS1 protein is a new common epitope region of dengue virus. *Jpn J Infect Dis* 64: 109–115.
34. Putnak JR, Charles PC, Padmanabhan R, Irie K, Hoke CH, Burke DS, 1988. Functional and antigenic domains of the dengue-2 virus nonstructural glycoprotein NS-1. *Virology* 163: 93–103.
35. Steidel M, Fragnoud R, Guillotte M, Roesch C, Michel S, Meunier T, Paranhos-Baccala G, Gervasi G, Bedin F, 2012. Nonstructural protein NS1 immunodominant epitope detected specifically in dengue virus infected material by a SELDI-TOF/MS based assay. *J Med Virol* 84: 490–499.
36. Wu H-C, Huang Y-L, Chao T-T, Jan J-T, Huang J-L, Chiang H-Y, King C-C, Shiao M-F, 2001. Identification of B-cell epitope of dengue virus type 1 and its application in diagnosis of patients. *J Clin Microbiol* 39: 977–982.
37. Yao ZJ, Kao MC, Loh KC, Chung MC, 1995. A serotype-specific epitope of dengue virus 1 identified by phage displayed random peptide library. *FEMS Microbiol Lett* 127: 93–98.
38. Chang H-H, Shyu H-F, Wang Y-M, Sun D-S, Shyu R-H, Tang S-S, Huang Y-S, 2002. Facilitation of cell adhesion by immobilized dengue viral nonstructural protein 1 (NS1): arginine-glycine-aspartic acid structural mimicry within the dengue viral NS1 antigen. *J Infect Dis* 186: 743–751.
39. Falconar AK, 1997. The dengue virus nonstructural-1 protein (NS1) generates antibodies to common epitopes on human blood clotting, integrin/adhesin proteins and binds to human endothelial cells: potential implications in haemorrhagic fever pathogenesis. *Arch Virol* 142: 897–916.
40. Immenschuh S, Rahayu P, Bayat B, Saragih H, Rachman A, Santoso S, 2013. Antibodies against dengue virus nonstructural protein-1 induce heme oxygenase-1 via a redox-dependent pathway in human endothelial cells. *Free Radic Biol Med* 54: 85–92.
41. Lin C-F, Lei H-Y, Shiao A-L, Liu H-S, Yeh T-M, Chen S-H, Liu C-C, Chiu S-C, Lin Y-S, 2002. Endothelial cell apoptosis induced by antibodies against dengue virus nonstructural protein 1 via production of nitric oxide. *J Immunol* 169: 657–664.
42. Khan AM, Heiny A, Lee KX, Srinivasan K, Tan TW, August JT, Brusic V, 2006. Large-scale analysis of antigenic diversity of T-cell epitopes in dengue virus. *BMC Bioinformatics* 7 (Suppl 5): S4.
43. Chung KM, Nybakken GE, Thompson BS, Engle MJ, Marri A, Fremont DH, Diamond MS, 2006. Antibodies against West Nile Virus nonstructural protein NS1 prevent lethal infection through Fcγ receptor-dependent and -independent mechanisms. *J Virol* 80: 1340–1351.
44. Muller DA, Young PR, 2013. The flavivirus NS1 protein: molecular and structural biology, immunology, role in pathogenesis and application as a diagnostic biomarker. *Antiviral Res* 98: 192–208.
45. Akey DL, Brown WC, Dutta S, Konwerski J, Jose J, Jurkiw TJ, DelProposto J, Ogata CM, Skiniotis G, Kuhn RJ, Smith JL, 2014. Flavivirus NS1 structures reveal surfaces for associations with membranes and the immune system. *Science* 343: 881–885.
46. Shakib F, Stanworth DR, 1980. Human IgG subclasses in health and disease. (A review). Part I. *Ric Clin Lab* 10: 463–479.
47. Bokisch VA, Top FH, Russell PK, Dixon FJ, Müller-Eberhard HJ, 1973. The potential pathogenic role of complement in dengue hemorrhagic shock syndrome. *N Engl J Med* 289: 996–1000.
48. Thein S, Aaskov J, Myint TT, Shwe TN, Saw TT, Zaw A, 1993. Changes in levels of anti-dengue virus IgG subclasses in patients with disease of varying severity. *J Med Virol* 40: 102–106.
49. Masrinoul P, Omokoko MD, Pambudi S, Ikuta K, Kurosu T, 2013. Serotype-specific anti-dengue virus NS1 mouse antibodies cross-react with prM and are potentially involved in virus production. *Viral Immunol* 26: 250–258.