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Original Article

Isolation and Characterization of Two Phenotypically Distinct Dengue Type-2 Virus Isolates from the Same Dengue Hemorrhagic Fever Patient

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SUMMARY: Dengue is the one of the most prevalent arthropod-borne viral diseases. Dengue virus circulates between humans and mosquitoes, and causes a wide range of disease in humans. To elucidate the link between the cell tropism of dengue virus and its pathogenesis, peripheral blood cells of infected patients were analyzed by flow cytometry. The dengue virus antigen was detected in peripheral $CD19^+$ cells (B cells) in one dengue hemorrhagic fever patient. Two dengue type-2 virus isolates were recovered from this patient using mosquito cell line C6/36 and human hematopoietic cell line K562, and designated VNHCM18-C/02 and VNHCM18-K/02, respectively. VNHCM18-K/02 exhibited strong binding ability and high infectivity to a B-lymphocyte cell line (RPMI8226) but showed poor growth in C6/36 cells, while VNHCM18-C/02 more efficiently and dominantly grew in C6/36 cells but did not efficiently bind to nor infect the B-cell line. Three amino acid differences were detected; one in an envelope protein (E-62) and two in nonstructural proteins. The distinct cell-binding to RPMI8226 was attributed to the difference between the two isolates in envelope protein E-62. Thus, we isolated two dengue type-2 virus variants with different cell-tropisms from the same patient, suggesting possible co-circulation in the patient.

INTRODUCTION

Dengue (DEN) virus belongs to the genus *Flavivirus* of the family *Flaviviridae*. Four antigenically distinct serotypes of DEN virus (DEN-1 to -4) circulate between humans and the mosquito vectors, *Aedes aegypti* and *Aedes albopictus* (1,2). DEN viruses cause a wide range of disease in humans, from acute febrile illness dengue fever (DF) to the more severe, life-threatening forms, dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), characterized by increased vascular permeability and hemorrhagic manifestations (3). Worldwide, DEN is one of the most prevalent arthropod-borne viral diseases and a leading cause of morbidity and mortality among children in tropical and subtropical regions (4). The number of DEN virus infections and the geographical spread to new areas of both vector mosquitoes and viruses have increased in recent decades, and currently more than 2.5 billion people live in areas where DEN virus infection is a risk. More than 50 million cases of infection occur every year, resulting in around 500,000 people developing DHF/DSS, with an average fatality rate of 1 - 5% (5-8).

DEN viruses are enveloped positive-sense single-stranded RNA viruses (~10.7 kb) whose genome encodes three structural proteins—capsid (C), premembrane (prM), and envelope (E)—and seven nonstructural proteins—NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5. Flavivirus virions are composed of a nucleocapsid core surrounded by a cell-derived lipid bilayer membrane and two envelope-associated glycoproteins, E and prM. E protein, the major virion surface protein, mediates virus attachment and membrane fusion. Interaction between the receptor-binding site(s) and receptors on a susceptible host cell allows entry into the cell by endocytosis. Flaviviruses utilize a variety of receptors for different cell types and host species (9), and a number of cell surface molecules have been identified as receptors for DEN virus (10). The interaction between E protein and these receptors on target cells is responsible for DEN virus infectivity.

Generally, once the DEN virus is transmitted by the bite of an infected mosquito into humans, mononuclear phagocytes such as dendritic cells (DCs), macrophages, and monocytes are considered to be the primary targets of DEN virus replication. This has been demonstrated in clinical and histopathological studies (11-14) and in vitro studies of DEN virus-permissive cells (15,16), as well as in studies using an immunodeficient murine model in vivo (17). However, only a few clinical studies have been conducted to identify the target cells of DEN virus infection in vivo, in view of the low frequency of virus detection in autopsies, as well as the limited range of specimens being focused on. Thus, the cell tropism of DEN virus in vivo remains poorly understood.

To elucidate the link between the cell tropism of DEN virus

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and its pathogenesis, we attempted to identify DEN virus-infected cells in the peripheral blood of DEN patients by flow cytometry, and to isolate DEN variants from patient serum using several cell lines. The isolates were characterized by their phenotypes. In this report, we demonstrate the differing cell tropisms of two DEN variants isolated from the same DEN patient, using two different cell lines, one from mosquitoes and the other from human blood.

PATIENTS AND METHODS

Patients: From September 2001 to September 2002 at National Children's Hospital No.1, Ho Chi Minh City, Vietnam, 80 blood samples were collected from admitted patients clinically diagnosed with DEN. The study protocol was approved by the Scientific and Ethics Committee of the Pasteur Institute, Ho Chi Minh City, and the Ethics Committee of the Institute of Tropical Medicine, Nagasaki University. Informed consent was obtained from all adult patients, and from the parents or guardians of all pediatric patients. The diagnoses of DEN primary and secondary infection and their clinical severity were based on WHO criteria (3). All serum samples were examined by DEN RT-PCR (18) for serotyping, and IgM-capture ELISA (19) for serological confirmation. Ten DEN-2 and nine DEN-4 were identified in 19 clinical cases by RT-PCR.

Cell lines: *A. albopictus* mosquito C6/36 cells (20) were cultured as described previously (21). Human blood cells and cell lines including hematopoietic K562 cells, K562/3 cells and the B lymphocyte cell lines Daudi, Raji and RPMI8226, were cultured in RPMI1640 medium (Gibco-BRL, Gaithersberg, Md., USA), supplemented with 10% FCS and penicillin-streptomycin and incubated at 37°C, in 5% CO_2. Later, K562/3 cells, a stable cloned cell line established in our laboratory by the limiting dilution method from K562 cells, were chosen for use in further propagation of virus isolates because of their high susceptibility to DEN virus (manuscript in preparation). To isolate primary B cells from peripheral blood mononuclear cells (PBMCs) of healthy donors, plasma and granulocytes were removed by gradient centrifugation using Lymphoprep™ (Axis-shield PoC AS, Oslo, Norway), and primary B cells were obtained through magnetic positive selection using CD19 microbeads (MACS Miltenyi Biotec, Bergisch Gladbach, Germany).

Virus isolation: Two virus isolates from the serum of an infant patient were used. The serum was inoculated into a monolayer culture of the C6/36 cell line, incubated in 2% FCS-MEM, or human blood cells and cell lines, incubated in 2% FCS-RPMI1640. Passage intervals were 6 to 7 days and 4 to 5 days, respectively.

Antibodies: Two mouse monoclonal antibodies (mAbs) against Flavivirus group-specific E glycoprotein, 6B6C-1 (isotype: IgG2a) (22) and 12D11/7E8 (isotype: IgG1), were used to detect DEN virus in the focus assay and flow cytometry. The mAbs were conjugated with HRP (Sigma, St. Louis, Mo., USA) and labeled with Alexa-488 (Molecular Probes, Eugene, Oreg., USA). Phycoerythrin (PE)-labeled mAbs against cell surface markers, i.e., CD3 (T lymphocytes), CD14 (monocytes), CD16 (NK cells) and CD19 (B lymphocytes), and all isotype controls, were obtained from Beckman Coulter (Fullerton, Calif., USA).

Surface and intracellular detection of virus antigen by flow cytometry: DEN RT-PCR-positive blood specimens were analyzed by two-color staining flow cytometry assay. Heparinized whole blood cells were fractionated and incubated with PE-labeled mAb against the cell surface marker on ice for 30 min. Blood cells or cells infected with DEN virus in vitro were then washed with PBS and fixed and permeabilized with IC Fix™ and IC Perm™ buffers (BioSource, Camarillo, Calif., USA), according to the manufacturer's instructions, and incubated on ice for 30 min with Alexa-488 labeled mAb 6B6C-1 or 12D11/7E8. Cells were washed twice in Perm buffer and resuspended in PBS. DEN antigens on the surface and inside the cells were analyzed by FACSCalibur™ and CellQuest software (Becton Dickinson, Sparks, Md., USA).

Sequence analysis: The genomes of DEN isolates were sequenced, with the exception of the 19 and 58 bases at the 5′ and 3′ termini of the genome, respectively. Viral RNA was extracted from infected culture fluid (ICF) using the QIAamp viral RNA mini kit (Qiagen, Valencia, Calif., USA), according to the manufacturer's instructions. Primers for RT-PCR and sequencing (primer sequences available upon request) were designed on the basis of the published sequence of DEN-2 strain 16681 (GenBank accession no. U87411). Reverse transcription was performed with Superscript III reverse transcriptase (Invitrogen, Carlsbad, Calif., USA) using a 3′ virus-specific primer, followed by PCR to amplify five overlapping cDNA fragments containing genome nucleotide regions 1 - 2443, 2112 - 3838, 3689 - 5883, 5382 - 8217, 8032 - 10696 with LA Taq DNA polymerase (TaKaRa Bio, Shiga, Japan). The DNA fragments were directly sequenced and analyzed using ABI PRISM Big Dye Terminator v1.1 cycle sequencing kits and ABI PRISM 3100-avant Genetic Analyzer (Applied Biosystems, Foster City, Calif., USA). The GenBank/EMBL/DDBJ accession numbers for the consensus sequences reported in this paper are AB479041 - AB479042.

Antigen-detection ELISA: The viral antigen was quantified by the micro-plate method as described previously (23). ELISA units of sample ICFs were estimated by comparing their optical density (OD) with those of serially diluted standard positive antigens with a predetermined endpoint titer (24). Briefly, 96-well microplates were coated with anti Flavivirus IgG (20 μl/ml), obtained by purification from DEN patients' pooled serum, and then blocked with BlockAce (Dainippon Sumitomo Pharma, Osaka, Japan). The DEN ICFs were added, the plates were washed, and HRP-conjugated mAb 12D11/7E8 was added. The HRP reaction was detected by adding *o*-phenylenediamine dihydrochloride (OPD) substrate in the presence of 0.02% H_2O_2, for 30 min at room temperature, and stopped with 1 N H_2SO_4; then the OD was measured at 492 nm.

Focus assay in C6/36 cells: Titer of DEN virus were determined by a focus-forming assay on a C6/36 cell monolayer. Serial dilutions of DEN viruses were inoculated to the cells, which were then incubated at 28°C with 5% CO_2 for 2 h and overlaid with MEM with 1% FCS containing 0.5% methylcellulose 4000. The plates were incubated at 28°C, 5% CO_2, for 4 days. To detect intracellular DEN virus antigens, the cells were fixed with 4% paraformaldehyde phosphate buffer, permeabilized with 1% Nonidet P-40 in PBS and stained with mAb 12D11/7E8. This was followed by adding HRP-conjugated goat anti mouse IgG (1:500 dilution; American Qualex, San Clemente, Calif., USA) and stained with DAB (Dojindo, Kumamoto, Japan) with 0.02% H_2O_2 in PBS. After 10 - 15 min incubation at room temperature, the plate was rinsed with tap water to terminate the color reaction. The stained foci were counted under a microscope to calculate

the FFU/ml as the virus titer. The detection limit of the focus forming assay was 10^2 FFU/ml.

Virus infection and analysis of virus growth: The RPMI8226 cells (2×10^5) were exposed to DEN virus for 2 h at 37°C and, subsequently, washed three times with RPMI1640 medium to remove excess virus, resuspended in 2% FCS-RPMI1640, and incubated until the day of assay. The cells were stained with mAb 12D11/7E8 and analyzed by flow cytometry, as described above. C6/36 cells (5×10^6) were infected with DEN virus at a multiplicity of infection (MOI) of 0.01 FFU for 2 h at 28°C. After infection, the cells were washed three times with FCS-free MEM and re-suspended in 5 ml of 2% FCS-MEM (1×10^6/ml), then placed in a 25-cm^2 culture flask and incubated, in duplicate, at 28°C. Supernatants were harvested daily for 9 days to determine the virus titer by focus assay using C6/36 cells.

DEN virus binding assay: RPMI8226 cells (2×10^5) were washed with cold Mg^{2+}/Ca^{2+}-free Hanks' Balanced Salt Solution (HBSS, pH 7.0) (Gibco) and incubated with 50 ELISA units (approximately 10^6 FFU in ICFs) of DEN virus for 1 h on ice (pH 7.0 - 7.2). The cells were then washed three times with cold HBSS containing 0.5% BSA and incubated with Alexa-488 labeled mAb for 30 min on ice. The cells were washed three times with HBSS and resuspended in PBS. DEN virus binding was analyzed by FACSCalibur™.

Protein modeling: Protein homology modeling was performed using the Molecular Operating Environment software (MOE ver. 2008.10) distributed by the Chemical Computing Group, and was based on the sequence data for the E protein of isolate VNHCM18-C/02 (E-62Glu), with recursive energy minimization using the DEN E protein pdb coordinate file 1OAN (RCSB Protein data bank) as a template. The homology model produced was then mutated and energy minimized to generate the VNHCM18-K/02 (E-62Lys). Visualization of the electrostatic potential and local electrostatic map prediction were computed after energy minimization in the MOE Software with rendering of ribbon and surface projections in Discovery Studio ViewerLite version 5.0 (Accelrys Corp., San Diego, Calif., USA).

RESULTS

Profile of DEN virus positive PBMC and virus isolation: PBMCs from 19 RT-PCR-positive patients were double-stained with mAbs (anti-CD mAb and anti-Flavivirus envelope mAb, 6B6C-1) and subjected to flow cytometry assay in order to identify DEN virus-infected cells in the patients' peripheral blood. DEN virus antigen was detected in the PBMCs of only one patient with DEN-2 infection. The patient was an 8-month-old male infant, classified as DHF grade I, primary infection, at 3 days after fever onset. Approximately 59% of B ($CD19^+$) cells in the PBMCs from the patient were positive for DEN virus antigens (Fig. 1C), but no positivity was found in the $CD3^+$ (Fig. 1B), $CD14^+$ or $CD16^+$ cells of the patient (data not shown).

The patient's serum was inoculated into C6/36 cells, K562 cells, two B cell lines (Daudi and Raji) and primary PBL-B cells. Two DEN-2 isolates were recovered from the first passage in K562 cells and the second passage in C6/36 cells, and were designated VNHCM18-K/02 (VN18-K) and VNHCM18-C/02 (VN18-C), respectively.

Molecular characterization of VN18-C and VN18-K: The genome nucleotide and deduced amino acid sequences of VN18-C and VN18-K primary isolates were compared.

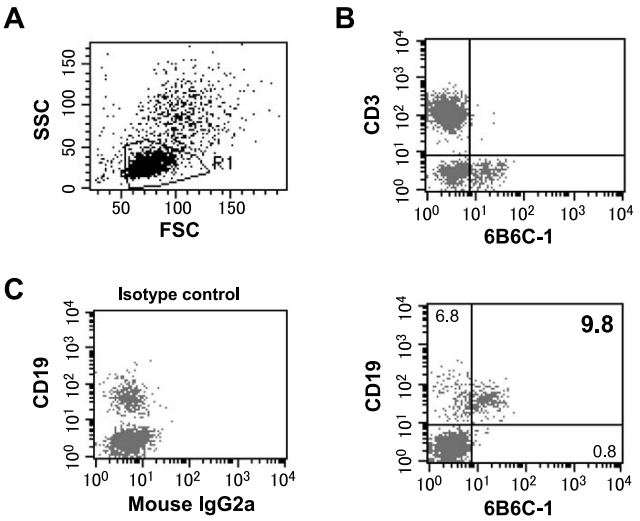

Fig. 1. Dot plot showing DEN virus antigen expression in PBMCs. PBMCs from a Vietnamese DEN patient were double stained with Alexa-488 labeled mAb (isotype control and anti-DEN E [6B6C-1]) and PE-labeled mAb to cell subset marker CD3 (T cells) and CD19 (B cells). (A) Dot plot represents lymphocyte gate in cell population size (FSC) versus cell granularity (SSC). (B) PBMCs stained with anti-CD3 mAb (*y* axis) and anti-DEN E mAb (*x* axis). DEN virus antigen in the lymphocyte gate was in $CD3^-$ cells. (C) PBMCs in the lymphocyte gate stained with anti-CD19 mAb (*y* axis), and anti-mouse IgG2a (left, *x* axis) or anti-DEN E mAb (right, *x* axis).

Table 1. Sequence variations in amino acids and nucleotides of the coding region identified in two isolates and following cross-passaged variants

Amino acid position	E-62	E-397	NS2B-114	NS4B-115
Nucleotide position[1)]	1120	2127	4472	7169
virus				
VN18-C	Glu GAG	Ser TCC	Ile ATA	Val GTA
VN18-K	Lys AAG	Ser TCT	Ile/Thr A(T/C)[2)]A	Ala GCA
variant				
VN18-CK	Lys AAG	Ser TCT	Ile ATA	Ala GCA
VN18-KC	Lys>Glu (A>G)[3)]AG	Ser TC(T>C)[3)]	Ile>Thr A(T>C)[3)]A	Ala>Val G(C>T)[3)]A

[1)]: Nucleotide at the position underlined.
[2)]: The presence of equal amounts of two genome populations of distinct nucleotides is indicated in parentheses.
[3)]: The appearance of a small nucleotide population based on sequencing chromatographic signals.

Differences in nucleotides and amino acids between the two isolates are shown in Table 1. Three amino acid variations were observed, one in the structural E protein at E-62 and two in nonstructural proteins at NS2B-114 and NS4B-115; in addition, one silent nucleotide alteration was observed at E-397. Interestingly, a sequencing chromatogram indicated that the nucleotide at nt 4472 of VN18-K isolate was actually a mixture of T and C, resulting in Ile/Thr at amino acid position NS2B-114 (Table 1). At least three populations of variants thus existed in the patient's serum.

Growth and focus morphology in C6/36 cells: The growth curves of VN18-C and VN18-K in C6/36 cells are shown in Fig. 2A. The cells were inoculated with each isolate at an MOI of 0.01. Although both isolates replicated in C6/36 cells, the virus growth patterns differed between the two. VN18-C grew faster than VN18-K throughout the time

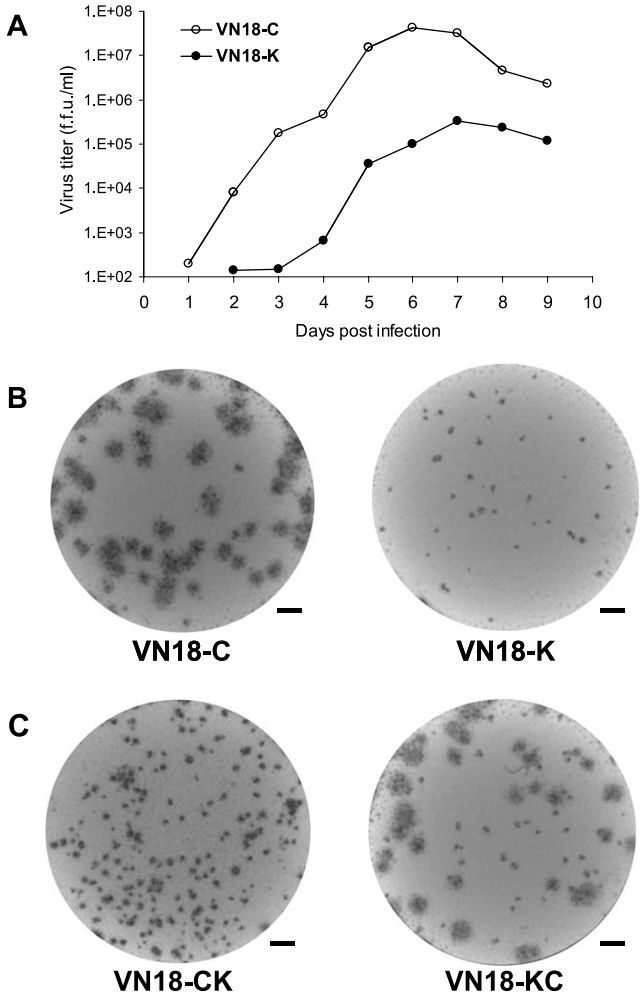

Fig. 2. (A) Comparison of the growth curves of VN18-C and VN18-K. Mosquito C6/36 cell lines were infected with either isolate at an MOI of 0.01, and supernatants were collected daily for 9 days. Virus titers were determined by focus assay in C6/36 cells. The graph represents the mean virus titer from two independent experiments. (B-C) Focus morphologies of the two isolates, VN18-C and VN18-K, were compared (B), and their cross-cell passaged variants VN18-CK and VN18-KC (C), at 4 days after virus inoculation in C6/36 cells. Bar, 1 mm.

course. VN18-C was detected from 1 day postinfection (p.i.) and reached 4.1×10^7 FFU/ml at 6 days p.i. In contrast, VN18-K was detected from 2 days p.i. and reached 3.4×10^5 FFU/ml at 7 days p.i. Thus, VN18-C virus could grow approximately 100-fold higher than VN18-K in C6/36 cells.

The focus morphologies of VN18-C and VN18-K in C6/36 cells were also compared (Fig. 2B). VN18-C exhibited mostly large foci (0.6 - 1.0 mm in diameter) with a few small foci (0.2 - 0.3 mm in diameter). In contrast, VN18-K exhibited only small foci (0.2 - 0.3 mm in diameter). The results of virus growth curves and the focus morphology analysis suggested that the VN18-C isolate grew more rapidly than the VN18-K isolate in the mosquito-derived C6/36 cells.

Cross-passaging of VN18-C and VN18-K isolates: In order to clarify the DEN virus variant selection mechanism in the cells, VN18-C virus isolated in C6/36 cells was inoculated into K562/3 cells at an MOI of 0.1 to 1, and monitored for 9 days. During incubation, three-quarters of the old medium were replaced with fresh medium every 4 or 5 days until the antigen titer reached a detectable level. Virus antigen was detected at 8 days p.i. and harvested at 9 days p.i. The ICF was designated VN18-CK. Foci of the VN18-CK in C6/36 cells were of a small size, similar to those of VN18-K, as shown in Fig. 2C. The nucleotide sequence of VN18-CK (Table 1) was identical to VN18-K, including a silent variation at 2127-T, suggesting that this K562/3 cell-adapted variant (VN18-CK) was not newly generated by mutation but was selected from a pre-existing VN18-K-type variant in the VN18-C isolate, and became a majority when passaged in K562/3 cells.

We also cross-cultured the VN18-K virus isolate in C6/36 cells twice and designated the resulting ICF VN18-KC. The foci of VN18-KC were both small and large (Fig. 2C). The nucleotide-sequencing chromatograms of VN18-KC showed the appearance of VN18-C-type nucleotide sequences at nt 1120, nt 2127, and nt 7169, including the silent variation (Table 1).

Infectivity assay in a B cell line, RPMI8226: As these DEN isolates had been recovered from the patient whose B cells were positive for DEN virus antigen, the infectivity profiles of VN18-C and VN18-K in the B cell line RPMI8226 were examined. RPMI8226 cells were infected with VN18-C and VN18-K at MOIs of 0.1, 1, and 5. The DEN virus ICFs were harvested at 12, 24, 48, 72 and 96 h p.i. in order to determine the virus titer by focus assay using C6/36 cells (Fig. 3A). Virus growth was observed from 24 and 48 h p.i. in VN18-K infection and reached peak titers of 2.5×10^3 and 1.2×10^5 FFU/ml at 48 h p.i. at MOIs of 0.1 and 1, respectively, and of 1.8×10^5 FFU/ml at 24 h p.i. at an MOI of 5. By contrast, no progeny virus production was observed in VN18-C-inoculated RPMI8226 cells up to 96 h p.i. The results by flow cytometry analysis for the infected cells at 24 h p.i. are shown in Fig. 3B. Approximately 90% of the cells were antigen positive at an MOI of 5 in VN18-K-inoculated cells, while no significant signal was detected from the VN18-C-inoculated cells.

We also examined the infectivity of VN18-C and VN18-K in several other B-cell lines: Daudi, Raji, Ramos, RPMI1788, and IM-9. Although—with the exception of RPMI1788 cells—these cell lines are known to be susceptible to DEN virus infection (25), none could be infected with either VN18-K or VN18-C, suggesting a very narrow human cell tropism in our two isolates.

Binding assay of VN18-C and VN18-K to RPMI8226 cells: In order to clarify the different infectivity between the two isolates, we performed a binding assay to determine whether the two isolates could adsorb RPMI8226 cells. Fifty ELISA units of VN18-C or VN18-K were incubated with the cells for 1 h on ice, and the cells were stained with Alexa-488 labeled mAb 12D11/7E8. Viruses binding to the cells were detected by flow cytometry. As expected, VN18-K bound to RPMI8226 cells, while VN18-C showed no specific binding (Fig. 4), suggesting that the binding capability is one factor determining the cell tropism of VN18-K.

The location of amino acid variation in a 3D model of the E protein: To further understand the difference in binding ability between the two isolates, the three-dimensional structures of the E protein were compared. The homology structure of DEN-2 isolate E protein was modeled based on the known structure of the DEN-2 virus (26). The location of the amino acid residue E-62 was in domain II, and very close to the Chiral Axis of the E protein dimer (Fig. 5A). The difference in residue 62 between the two isolates, acidic Glu in VN18-C and basic Lys in VN18-K virus, results in a local change in the electrostatic potential (Figs. 5B and 5C). The positively charged basic 62Lys protrudes slightly towards its

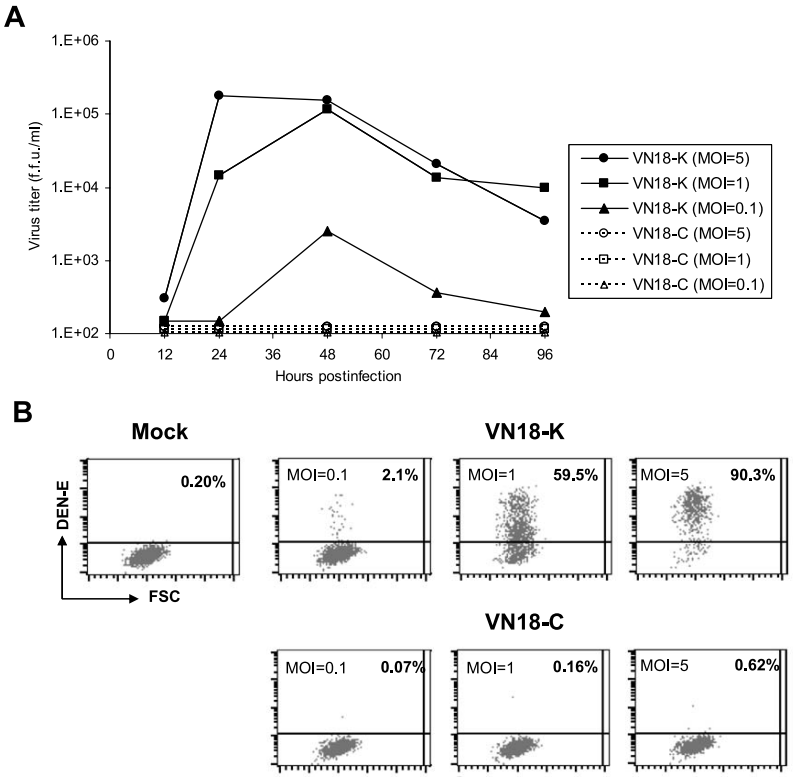

Fig. 3. Infectivity assay of DEN isolates in a B cell line. RPMI8226 cells were infected with VN18-K or VN18-C at MOI of 0.1, 1, and 5. (A) The ICFs were harvested at 12, 24, 48, and 96 h p.i. The titer of infectious virus in the supernatant was determined by focus assay. (B) DEN virus infected cells were fixed after the incubation, stained with Alexa-488-labeled mAb 12D11/7E8, and analyzed by flow cytometry. The results show percentages of anti-DEN E staining on RPMI8226 cells at 24 h after infection with VN18-K (upper) and VN18-C (lower). Mock (MOI = 0) infected cells were analyzed as a control (left).

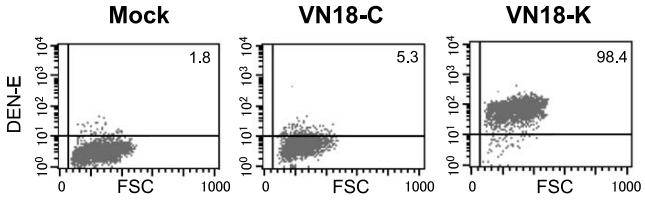

Fig. 4. Binding assay of VN18-C and VN18-K to RPMI8226 cells. RPMI8226 cells were mock treated or treated with 50 ELISA units of DEN antigens for 1 h on ice and stained with Alexa-488-labeled mAb 12D11/7E8, and analyzed by flow cytometry. The amount of DEN antigen was determined by antigen-detection ELISA. The results show percentages of virus-binding cells.

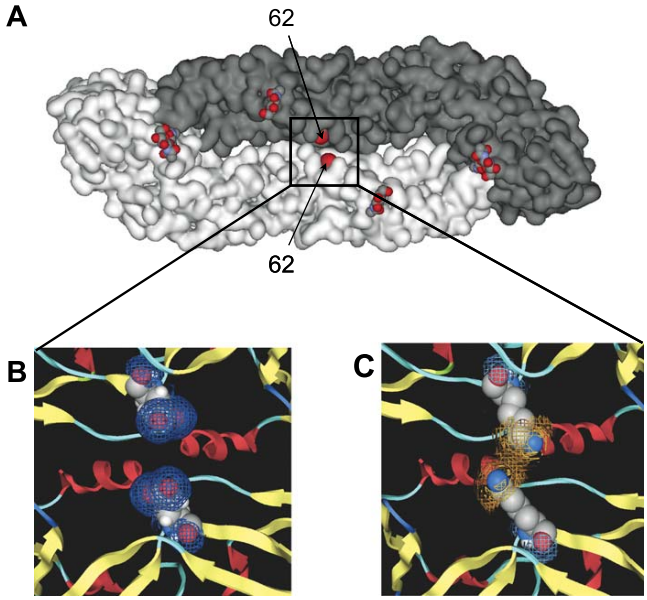

Fig. 5. Structure model of DEN-2 E protein dimer of two DEN-2 isolates. (A) The location of amino acid residue 62 is shown in a rendered surface representation of E protein dimer as viewed perpendicularly. (B-C) shows a close-up of the residue 62 area in panel A. Amino acid residue 62 is represented as space-filled in a ribbon diagram, 62Glu in VN18-C (B) and 62Lys in VN18-K (C). The meshes represent the immediate electrostatic field calculation of the molecules' local charge at the end of the amino acid.

equivalent amino acid in the neighboring E protein (Fig. 5C), because Lys has a longer carbon chain than Glu, resulting in an increase in its size.

DISCUSSION

It has been reported that the DEN virus exhibits a diversity of virus genome sequences within an individual host, as well as among hosts. The resulting virus populations are known as quasispecies (27-31). In this paper, we isolated two DEN-2 virus variants with different cell-tropisms (VN18-C and VN18-K, Table 1) from a single DHF patient. Although it is possible that DEN virus adaptation to cells could occur during passaging in vitro (32), it is very unlikely that the recovery of these variants (VN18-C and VN18-K) was the result of the emergence of an adaptive mutation during virus isolation because the VN18-CK (Table 1) obtained from VN18-C isolate after a single passage showed a sequence identical to that of VN18-K, including the silent variation (E-397), suggesting that the VN18-K-type virus certainly existed in the original patient serum as well as in the VN18-C culture fluid as a

minority population, and was selected during the passage (Fig. 2C). This phenomenon occurs in reverse for VN18-KC and VN18-C (Table 1). Moreover, we speculate that VN18-K was more dominant in the patient's serum than VN18-C, due to the fact that VN18-K was isolated in the first inoculation of serum to K562 cells as early as 5 days p.i., while VN18-C was identified only after the second passage in C6/36 cells, despite its rapid growth (Fig. 2). This could have been confirmed by the sequencing of cloned viral cDNA directly generated from the patient's serum RNA: however, we unfortunately did not have a sufficient volume of serum for this purpose.

DEN virus is capable of infecting a wide range of target cells in vivo and in vitro, such as DCs, macrophages, and monocytes, and also B lymphocytes, hepatocytes, and endothelial cells (12,14,25,33-41). Among these, DCs are considered to be the primary target cells that propagate infection to other more abundant target cells/organs in humans (12); however, the subsequent DEN virus targets have not been fully elucidated. King et al. (37) reported that DEN virus was recovered mainly from B lymphocytes, and that the DEN virus antigen was located intracellularly and on the surface of B lymphocytes in vivo. Jessie et al. (14) later demonstrated DEN virus antigen and RNA in the lymphocytes, macrophages, monocytes, and endothelial cells of autopsy samples by immunohistochemistry and in situ hybridization. In our present study, we detected DEN virus antigen in peripheral B lymphocytes in a DHF patient at a high level of positivity (59% of peripheral $CD19^+$ cells, Fig. 1C) by flow cytometry analysis, and isolated a virus, VN18-K, capable of infecting and effectively multiplying in a B cell line, RPMI8226. In addition, out of 25 DEN RT-PCR positives among 121 DEN patients enrolled in 1999 and in 2006 in the Philippines, flow cytometry detected DEN virus antigens in B cells of 7 cases, NK cells in 2 cases and both NK and B cells in 1 case. Among these 8 cases, 1 was identified DEN-1 and 7 as DEN-3. Further the DEN virus was isolated from 5 of the above-mentioned 8 cases (manuscript in preparation). Based on these and previous results, B cells can be associated and/or infected with DEN virus type-1, -2, and -3, and probably -4 as well. We therefore speculated we had isolated a B cell-tropic DEN virus. However, we could not successfully confirm the tropism of VN18-K in human B cells. We obtained B cells from 8 healthy adult volunteers and performed a virus infection assay. None of the 8 lots of B cells exhibited virus replication, as determined by flow cytometry. There are several possible explanations for this discrepancy: (i) another variant may have existed and been associated with the $CD19^+$ cells in our patient, (ii) infant B cells, or the B cells of this particular infant, may have possessed a specific receptor for VN18-K virus, or (iii) some specific condition could induce B cells to express the receptor molecule for VN18-K virus. Currently, we are attempting to identify the VN18-K virus receptor molecule(s) on K562/3 and RPMI8226 cells for further investigation.

The E protein forms head-to-tail homodimers in which each monomer has three domains lying on the viral envelope (42,43), and is a critical determinant of cell tropism and viral entry (9). Between VN18-C and VN18-K, there was a single amino acid difference at residue 62 in the E protein. VN18-K, which had 62Lys showed strong binding ability and infectivity in the B cell line PRMI8226, while VN18-C, with Glu at the same position, showed no binding ability or infectivity in this cell line (Figs. 3 and 4). 3D protein modeling analysis (Fig. 5) revealed that residue 62 was partially enclosed in a pocket at the interface between the E protein dimer pair; therefore, it is unlikely that it participates directly in binding to the cells. This special location of E-62 in domain II allows it the unique opportunity to auto-interact with the same amino acid on the other half of the E-dimer. It can be assumed that the Lys at position E-62 causes a higher electrostatic autorepulsive event than Glu due to the proximity of two identical paired amino acids. Even though the individual repulsive force of the interaction between the E-62 pair is small (ranging from 3 to 6 Kcal/mol), the net combination of these repulsions may lead to a dimer-loosening effect throughout the surface of the virus particle on which 90 E-dimers are located. This dimer-loosening effect of the E protein would appear to be sufficient to allow the lysine-bearing VN18-K easier access to the receptor-binding site in the hydrophobic pocket of the cell surface receptor, and result in the net increased binding ability of the virus. Thus, we demonstrated that the difference in binding ability to RPMI8226 cells could be attributed to the E-62 residue.

As shown in Table 1, amino acid differences were also found in the nonstructural proteins NS2B (a possible cofactor of NS3 serine protease activity) (44) and NS4B (an inhibitor of IFN signal transduction in the host cell) (45-47), between the two isolates; i.e., NS2B-114Ile and NS4B-115Val, and NS2B-114Thr/Ile and NS4B-115Ala, respectively. These differences in the NS proteins may also play a role in determining the different phenotypes of variants in human cell lines and mosquito cell lines, for example the better growth of VN18-C in C6/36 cells than in K562/3 cells (Fig. 2). However, the role of the amino acid differences in the nonstructural proteins is still unclear, and further studies using DEN virus infectious cDNA clones are required.

DEN virus and other arboviruses have life cycles that uniquely equip them for survival in nature. DEN virus sequentially infects humans and mosquitoes, quite disparate hosts. Diversity among DEN virus genomes and phenotypes may play an important role in the survival of DEN viruses in very different cell environments. In the present study, we observed that a given type of variant readily becomes the dominant population in susceptible cells, such as VN18-C in the mosquito cell line and VN18-K in human blood cell lines. Accordingly, population switching of DEN variants was observed between C6/36 cells and K562/3. However, it was noted that the variants can also survive in less susceptible cells by co-existing with the type of variant dominant in that cell type. This observation may mimic events in nature, as the VN18-C type is dominant in mosquitoes and the VN18-K type is dominant in humans. Each type may help the survival of the other and in turn may facilitate effective multiplication and transmission of DEN virus in both mosquitoes and humans. Actual rescue among DEN virus variants in nature has been demonstrated by Aaskov et al. (48). Studying DEN epidemics in Myanmar, they found that defective DEN virus with a stop-codon in its E protein could be transmitted to and maintained in humans and mosquitoes through complementation with co-existing functional viruses. Taken together, this information suggests that DEN virus population switching occurring between humans and mosquitoes and the resulting heterogeneity of the DEN virus population may facilitate the efficient survival of the DEN virus in both humans and mosquitoes.

The severity of DEN virus infection is considered to be related to many factors, including virus factors (27,49), antibody-dependent enhancement (50,51), host genetic fac-

tors (52,53), and the cellular immune response (51,54). Due to the lack of appropriate animal models to mimic the myriad clinical symptoms of DEN, the virulence of VN18-C and VN18-K is difficult to assess. We believe that further clarification of DEN virus variants and their cell tropism is essential.

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