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Phenotypic analysis of dengue virus isolates associated with dengue fever and dengue hemorrhagic fever for cellular attachment, replication and interferon signaling ability

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ABSTRACT

Eighteen dengue viruses (DENVs) representing all four serotypes, isolated from pediatric patients at children's hospital, Queen Sirikit National Institute of Child Health, Bangkok, Thailand exhibiting a diverse spectrum of disease ranging from uncomplicated dengue fever (DF) to severe dengue hemorrhagic fever (DHF), were tested for their ability to attach to host cells, replicate and interfere with the IFNα signaling pathway by interfering with signal transducer and activator of transcription 1 (STAT-1) function. Although most isolates suppressed IFNα-induced STAT-1 phosphorylation, our results showed no difference between DENV strains associated with DF and those associated with DHF. However, the DHF isolates tended replicate to higher titers in dendritic cells (DCs) than the DF isolates, but this ability was independent of their cell-binding capability. Our results suggest that the emergence early in infection of viruses with a high degree of replication fitness may play an important role in DENV pathogenesis.

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1. Introduction

The dengue viruses (DENVs) are single-stranded, positive-sense RNA viruses in the family *Flaviviridae* and consist of four antigenically related but distinct serotypes denoted DENV-1 to DENV-4, which are transmitted to humans by mosquito vectors, primarily *Aedes aegypti*. These viruses have a genome of approximately 11 kb containing one open reading frame that encodes three structural proteins, capsid (C), pre-membrane/membrane (PrM/M), and envelope (E), and seven non-structural (NS) proteins, NS1, NS2A/2B, NS3, NS4A/4B, and NS5, flanked by 5′- and 3′-non-translated regions (5′NTR/3′NTR).

Dengue (DEN) has reached epidemic proportions in the tropics and subtropics and has even spread to some temperate regions with the result that it is now the most widespread and prevalent arthopod-borne viral disease of man. It is estimated that more than 100 million people are infected annually, typically resulting in a relatively mild, acute febrile illness called dengue fever (DF). However, some individuals, particularly those who have had a previous DENV infection, may go on to develop a more severe disease spectrum called dengue hemorrhagic fever (DHF) sometimes leading to dengue shock syndrome (DSS) (Halstead and Simasthien, 1970). Of the estimated one million cases of DHF/DSS per year, about 5% are fatal (Lei et al., 2001).

Epidemic transmission of DENV leading to large outbreaks of DF and DHF previously occurred about every 10–40 years; however, now large epidemics occur every 3–5 years (Cummings et al., 2004). The factors responsible for the increased frequency and size of DEN epidemics are not known. It can be speculated upon rising global temperature, changes in rainfall pattern and human/vector population density, rapid transportation, and virus strains may also play an important role. The data from epidemiological studies show that the introduction of genetically distinct Asian DENV-2 strains into the Americas was associated with an increase in DHF/DSS cases (Rico-Hesse et al., 1997). Furthermore, these newly introduced viruses exhibit changes, most notably within the E gene and the 5′NTR/3′NTR, which may correlate with increased disease severity (Mangada and Igarashi, 1997; Pandey and Igarashi, 2000). Nevertheless, it has not been possible to conclusively associate viral genotypic changes with changes in virus transmissibility and pathogenicity due to the lack of satisfactory *in vitro* or *in vivo* disease models. However, recent studies of DENV infection of pri-

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mary human dendritic cells (DCs), which are believed to play an important role in primary viral infection, demonstrate that DENV strains associated with more severe disease also exhibit a higher level of infectivity and cytokine induction more than attenuated strains (Lozach et al., 2005; Navarro-Sanchez et al., 2003; Sanchez et al., 2006; Wu et al., 2000). Therefore, these cells may provide a new tool to study the relationships between DENV genotype and disease severity.

All four DENV serotypes have been co-circulating in Thailand for many years. Results of DENV surveillance from children's hospital in the Queen Sirikit National Institute of Child Health (QSNICH), Bangkok show that the number of DF and DHF cases caused by each serotype increased dramatically after the 1990s (Nisalak et al., 2003). However, the results of our investigation of the molecular epidemiology of DENVs circulating in Bangkok area over the past three decades show that the predominant genotype for each serotype remained unchanged (Klungthong et al., 2004; Zhang et al., 2005; Zhang et al., 2006). This suggests that the observed increase in total DEN cases is not due to the introduction of new genotypes, but is attributable to existing strains. Comparative analysis of the complete genomic sequences of Thai DENV representative strains revealed significant amino acid (aa) mutations across almost the entire genome, particularly within the NS genes (unpublished data), which leads to the hypothesis that mutations in the NS genes might allow the virus to counter the host's antiviral responses, including the interferon-alpha (IFNα) response. Recent reports show that NS2A, NS4A, and especially NS4B of DENV play a role in inhibition of host interferon responses (Ho et al., 2005; Munoz-Jordan et al., 2005, 2003).

IFNα is a critical cytokine released by a variety of cell types including DCs and serves to limit viral replication prior to full recruitment of antigen-specific humoral and cell-mediated immune responses. The IFN-mediated antiviral responses occurs when IFNα produced from virus-infected cells binds to the IFNα receptor on the surface of infected or bystander cells, resulting in the activation of the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway. Once activated, phosphorylated STAT translocates to the nucleus where it binds promoter regions in consensus DNA-recognition sites for transcription of numerous IFN-stimulated genes and induction of antiviral responses. Many pathogenic viruses including DENV encode proteins that direct mechanisms to counter the IFN response (Ho et al., 2005; Jones et al., 2005; Munoz-Jordan et al., 2003; Samuel, 2001).

Prior to this study we speculated that the increased incidence of severe DENV illness might be related to mutations in viral NS proteins that antagonize host IFN pathways and allow the virus to partially escape the host's immune defense system. A comparison of the sequences of DENV strains isolated from DF and DHF patients reveals genetic changes within the E gene that might enhance virus attachment to and replication in host cells (Bennett et al., 2006). In the present study we found that while most of our DENV isolates suppressed IFNα-induced STAT-1 phosphorylation, there was no significant difference between the DF and DHF isolates in this regard. However, our results showed that the viral isolates from DHF patients exhibited higher levels of replication in human DCs than that from DF patients, although the increased replication rate was not associated with increased attachment of virus to the host cells.

2. Materials and methods

2.1. Viruses

Eighteen DENV isolates representing all four serotypes (Table 1) selected from a large DENV bank collected from the children's hospital of the QSNICH, Bangkok over the period 1981–2001, were tested in this study. All samples were from cases that had been clinically graded based on the WHO guidelines for DF and DHF/DSS and were well characterized both serologically and molecularly. These viruses were first amplified in *Toxorhynchites splendens* mosquitoes followed by one to three passages (7 days for each passage) in C6/36 mosquito cells, and then serotyped by both an antigen capture enzyme linked immunosorbent assay (ELISA) and a two step nested RT-PCR, and genotyped by phylogenetic analysis of the virus E gene sequences. The complete genomes of these selected trains representing each serotype and genotype were then sequenced and analyzed. The background epidemiological and methodical data are described elsewhere (Klungthong et al., 2004; Zhang et al., 2005; Zhang et al., 2006). A brief description of 18 samples for the study and their accession numbers in GenBank are listed in Table 1. Viral infectivity titers were determined by the plaque forming unit (PFU) assay in Vero cells, and expressed as PFU/ml.

Table 1
Sample information of the 18 DENV isolates used in the comparative study.

Viruses[a]	Diseases	Infections	Sex	Age (years)	GenBank accession #
DENV-1					
ThD1-0008/81	DHF II	Primary	F	0.5	AY732483
ThD1-0081/82	DF	Primary	F	12	AY732481
ThD1-0323/91	DHF III	Primary	M	4	AY732478
ThD1-0097/94	DHF II	Secondary	M	10	AY732480
ThD1-0488/94	DF	Primary	M	4	AY732475
DENV-2					
ThD2-0498/84	DHF IV	Primary	M	14	DQ181804
ThD2-0433/85	DHF IV	Secondary	M	4	DQ181803
ThD2-0026/88	DF	Secondary	M	13	DQ181802
ThD2-0017/98	DF	Secondary	M	14	DQ181799
ThD2-0078/01	DHF III	Secondary	F	8	DQ181797
DENV-3					
ThD3-0055/93	DHF II	Primary	F	2	AY676351
ThD3-0104/93	DF	Secondary	F	13	AY676350
ThD3-1283/98	DF	Primary	M	3	AY676349
ThD3-1687/98	DHF III	Secondary	M	11	AY676348
DENV-4					
ThD4-0017/97	DF	Secondary	M	10	AY618989
ThD4-0476/97	DHF II	Secondary	F	8	AY618988
ThD4-0734/00	DF	Secondary	M	15	AY618993
ThD4-0485/01	DHF I	Secondary	M	6	AY618992

[a] Viruses used in this study were isolated from patients admitted to Children's Hospital, QSNICH, Bangkok, Thailand, graded as DF or DHF I to IV by experienced clinicians based on WHO's scale for DEN disease severity, and serologically characterized as primary or secondary infection.

2.2. Raji DC-SIGN transfected cells

DC-SIGN (*d*endritic *c*ell-specific *i*ntercellular adhesion molecule 3-grabbing *n*on-integrin) Raji cells are a Burkitt's lymphoma-derived lymphoblastoid cell line stably transfected with DC-SIGN, a C-type lectin known as a receptor for dengue virus binding, internalization and infection (Alen et al., 2009; Lozach et al., 2005; Marijke et al., 2009; Navarro-Sanchez et al., 2003; Tassaneetrithep et al., 2003). The cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine and 1% Penicillin/Streptomycin at 37 °C with 5% CO_2 and 95% relative humidity and used for cellular attachment studies.

2.3. Dendritic cells (DCs)

Peripheral blood mononuclear cells (PBMCs) were isolated from human whole-blood units of healthy, dengue seronegative con-

senting donors under a protocol approved by the Human Subjects Research Review Board (HSRRB) of the Walter Reed Army Institute of Research. Monocytes ($CD14^+$ cells) were separated from PBMCs by a magnetic column according to the manufacturer's instructions (Miltenyi Biotech, Inc., Auburn, CA). Monocyte-derived DCs were generated by culturing monocytes in RPMI 1640 medium supplemented with 10% FBS, 1% minimum essential amino acids, 1% L-glutamine, 1% sodium pyruvate, 1% Penicillin/Streptomycin, 50 μg/ml rhuIL-4 and 3.5 μg/ml rhuGM-CSF at 37 °C with 5% CO_2 and 95% relative humidity for 7 days (Palmer et al., 2005). The purity of cells was assessed by flow cytometry using a panel of antibodies known to target DCs markers including CD11c and CD1a (Palmer et al., 2007; Palmer et al., 2005; Sun et al., 2006). The majority of DCs (>95%) were $CD11c^+$ and $CD1a^+$, <1% of the cells expressed CD14. Monocytes do not survive the culture conditions outlined above and they could be excluded from the monocyte-derived DCs used in this study.

2.4. A Real time quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) assay

The primers and probes used to measure viral RNA copy numbers for specific DEN serotypes 1–4 were designed for a target in the C gene and described by Sadon et al. (2008). The copy number of viral RNA was calculated based on a known amount of standard viral RNA.

2.5. To measure the capability of virus binding

To determine whether DENV has the similar capability of binding in Raji DC-SIGN transfected cells as in the DCs, a DENV-2 laboratory strain (S16803) was tested first in both cells and then 18 Thai DENV strains were tested in Raji cells. The Raji and DCs were washed with ice cold 10% FBS/RPMI 1640 medium twice and then chilled on ice for 30 min. Cells at a concentration of 1.5×10^5 cells/ml were incubated with DENV at multiplicity of infection (MOI) = 1 PFU/cell for 1.5 hours (h) at 0 °C in serum-free RPMI 1640 medium containing 1% minimum essential amino acid, 1% L-glutamine, 1% sodium pyruvate, 1% Penicillin/Streptomycin with frequent shaking. Unbound viruses were removed by three washes in cold 5% FBS/RPMI 1640 medium, then cold 0.01 M phosphate buffered saline (PBS) only, followed by suspension in 200 μl of cold PBS. Half of the cells were treated with 0.5 μg/ml Proteinase K (Sigma–Aldrich, St. Louis, MO) at 0 °C for 1 h to remove cell surface bound DENV. Cells were washed with cold PBS three times and then bound viral RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. The total bound viral RNA copy number was measured by a real-time one-step qRT-PCR assay.

2.6. To determine virus replication efficiency in DCs

The DCs (1×10^6/ml) were infected with 18 DENV strains at MOI = 1 and incubated at 37 °C with 5% CO_2 for 2.5 h. Infected cells were washed twice with 10% FBS/RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 1% minimum essential amino acid, 1% L-glutamine, 1% sodium pyruvate and 1% Penicillin/Streptomycin to remove excess viruses. Virus supernatants were collected after 24, 48 and 72 h post infection. Virus RNA was extracted from 140 μl culture supernatant using a QIAmp Viral RNA Mini kit by following the manufacturer's instructions (Qiagen, Valencia, CA). The copy number of viral RNA was measured by a real-time one-step qRT-PCR assay using the QuantiTect Probe RT-PCR kit (Qiagen, Valencia, CA) and an ABI 7000 Sequence Detection System (Applied Biosystem, Foster City, CA).

2.7. To determine the inhibition of STAT-1 phosphorylation in DENV infected DCs

DCs (1×10^6/ml) were infected with seven DENV strains (S16803, ThD2-0026-88, ThD2-0433-85, ThD4-0017-97, ThD4-0476-97, ThD4-0734-00 and ThD4-0485-01) at MOI = 1 at 37 °C with 5% CO_2 for 2.5 h. Infected cells were washed twice with 10% FBS/RPMI 1640 medium (Invitrogen, Carlsbad, CA) to remove excess viruses. The viral RNA copy number in infected DCs was measured by the qRT-PCR assay. In some experiments, the ability of viral isolates to inhibit STAT-1 phosphorylation in DCs was assessed 24 h post infection. Cells were harvested after infection and then treated with 1000 U/ml IFNα (type I) (PBL Interferon Source, Piscataway, NJ) at 37 °C with 5% CO_2 for 1 h followed by lysing with 100 μl lysis buffer [10 mM Tris–Cl (pH 7.5), 1 mM EDTA, 2 mM dithiotheitol, 0.1% Triton X-100] and protease inhibitor cocktail (Sigma–Aldrich, St. Louis, MO). Cell lysates were clarified by centrifugation at 5000 rpm at 4 °C for 5 min and then the supernatants were collected for SDS-PAGE gel electrophoresis and immunoblotting. Briefly, equal volumes of lysates of DENV infected DCs were subjected to SDS-PAGE on a 8% gel then transferred to nitrocellulose membrane and analyzed by Western Blotting with rabbit anti-STAT-1, phospho-STAT-1 (Cell signaling technology Inc, Danvers, MA) at a dilution of 1:1000 or human GAPDH loading control antibody (Chemicon International Inc., Temecula, CA) at a dilution of 1:1000 followed by HRP-conjugated goat anti-rabbit secondary antibody (Cell signaling technology Inc. Danvers, MA) at a dilution of 1:1000. Membranes were incubated with ECL chemiluminescence substrate at room temperature for 5 min (Amersham GE, Buckinghamshire, UK) then exposed to hypersensitivity X-ray film (Amersham GE).

3. Results

3.1. Comparison of the cell-binding efficiency of DF- and DHF-associated DENV isolates to DCs and DC-SIGN transfected Raji cells

To determine whether the severity of disease associated with the different DENV isolates was correlated with the efficiency of viral attachment to host cells, we measured the binding capabilities of 18 DENV isolates (i.e., the eight DF-causing isolates and 10 DHF-causing isolates shown in Table 1) to DCs and DC-SIGN transfected Raji cells. As shown in Fig. 1, the laboratory DENV strain S16803 bound efficiently to both cell types. However, experiments conducted at 0 °C to limit virus internalization, followed by pro-

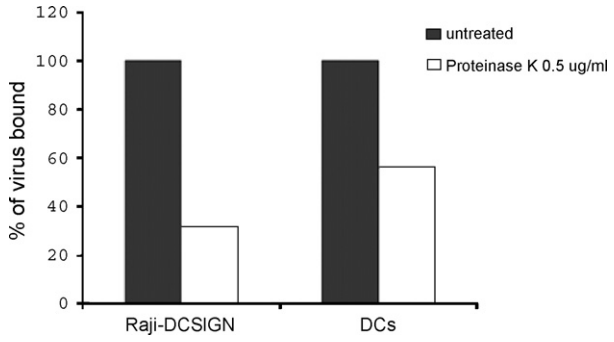


Fig. 1. Binding assay: Binding of laboratory DENV strain S16803 to DC-SIGN transfected Raji cells and human Dendritic (DCs). Cells were infected with DENV serotype type 2 (strain S16803) at 0 °C for 1.5 h. After excessive washes with PBS to remove unbound viruses, surface bound virus was determined by a real time qRT-PCR assay. Verification that virus particles were surface bound was obtained in concurrent experiments performed with cells treated with 0.5 μg/ml proteinase K for 1 h at 0 °C to remove bound viruses. The histograms shown represent one of three independent experiments.

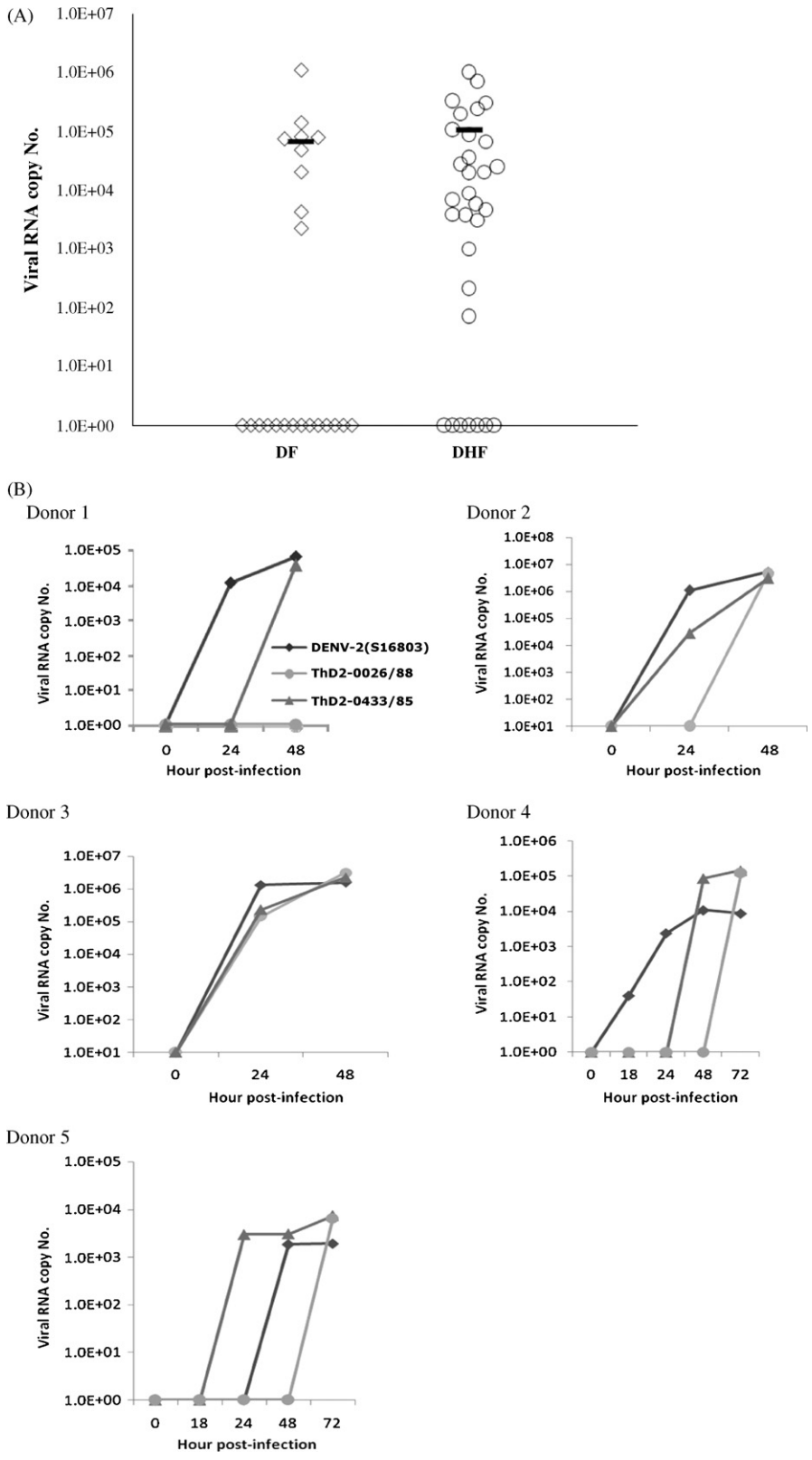


Fig. 2. (A) Mean viral RNA copy numbers were measured in 48 h post-infection supernatants collected from DF and DHF-infected human DCs from 3 to 5 donors (each bar represent mean ± SD for DF = $6.62 \times 10^4 \pm 2.24 \times 10^5$; DHF = $1.04 \times 10^5 \pm 2.26 \times 10^5$ viral RNA copy No. (B) Replication kinetics of DF isolate ThD2-0026-88 and DHF isolate ThD2-0433-85 in human DCs. Human DCs from five donors were infected with each isolate at an MOI of 1.0 and viral RNA copy numbers in harvested supernatants were measured at the indicated times by qRT-PCR.

Table 2
Binding assay of Thai DENV in Raji-DC-SIGN cells.

Disease severity	Dengue virus serotypes			
	1	2	3	4
DF	$1.75 \times 10^7 \pm 1.73 \times 10^7$	$1.02 \times 10^8 \pm 1.70 \times 10^8$	$8.11 \times 10^6 \pm 5.70 \times 10^5$	$3.19 \times 10^5 \pm 3.55 \times 10^5$
DHF	$1.99 \times 10^8 \pm 3.30 \times 10^8$	$3.46 \times 10^6 \pm 4.86 \times 10^6$	$3.53 \times 10^6 \pm 3.83 \times 10^6$	$1.92 \times 10^5 \pm 2.68 \times 10^5$

The same amount of each virus (MOI = 1) was incubated with Raji-DC-SIGN cells for 1.5 h at 0 °C. After five washes with PBS, cell-bound viruses were determined by a real time qRT-PCR assay. Each data set was from two independent experiments.

Table 3
The replication efficiency of DENV in human DCs.

Diseases severity	Dengue virus serotypes			
	1	2	3	4
DF	$2.16 \times 10^5 \pm 4.27 \times 10^5$	$1.41 \times 10^3 \pm 2.44 \times 10^3$	$1.56 \times 10^4 \pm 2.95 \times 10^4$	$1.60 \times 10^4 \pm 3.00 \times 10^4$
DHF	$2.89 \times 10^5 \pm 3.82 \times 10^5$	$5.44 \times 10^4 \pm 1.08 \times 10^5$	$5.04 \times 10^4 \pm 9.81 \times 10^4$	$2.28 \times 10^4 \pm 2.28 \times 10^5$

The replication efficiency of DENV in human DCs was determined as viral RNA copy number from 48 h p.i. supernatant of DENV-infected DCs (MOI = 1). The replication efficiency of each isolate was tested in human DCs from 3 to 5 different donors.

teinase K treatment to detach bound virus, showed that relatively more viruses bound to DC-SIGN transfected Raji cells (70%) compared to DCs (44%). This result might indicate greater variability of DC-SIGN expression on human DCs, which were obtained from different donors. Therefore, all subsequent measurements of the cell-binding capability of 18 DENV isolates were carried out in DC-SIGN transfected Raji cells and the amount of bound viruses was determined by real time qRT-PCR assay. Although the isolates varied in the ability to bind to the DC-SIGN transfected Raji cells (Table 2), there was no significant difference in the cell attaching capability of DF and DHF-associated virus strains.

3.2. Comparison of the replication efficiency of DF- and DHF-associated viral isolates in human DCs

We then evaluated the replication kinetics of the DF- and DHF-associated viral isolates in human DCs. The amount of virus produced in the infected cells by DF- and DHF-associated isolates was measured in supernatants collected from infected cultures at 48 h post-infection by real time qRT-PCR assay, and the average RNA copy numbers were compared with each other. As shown in Fig. 2(A) and Table 3, viruses isolated from DHF patients replicated more efficiently in human DCs than that of DF isolates. It is also worth noting that the DHF isolates within each serotype produced more viral progeny than DF isolates of the same serotype (Table 3).

3.3. Comparison of the replication of DF isolate (ThD2-0026-88) and DHF isolate (ThD2-0433-85) in human DCs

Previous reports have stated that some strains of the DENV-2 are associated with DHF. Our data on replication efficiency of DENV-2 presented in Table 3 showed that viruses isolated from the DHF cases had high replication efficiency in DCs than viruses isolated from DF cases. We, therefore, further examined the replication kinetics of DF and DHF isolates in DC cultures derived from five different donors. One pair of viral isolates from male patients with secondary DENV infection admitted to QSNICH during the 1985–88 outbreaks were selected for comparison analysis. Isolate ThD2-0433-85 was from a patient with DHF level IV and ThD2-0026-88 was from a patient with uncomplicated DF. Alignment analysis of the aa sequences of these two viruses revealed that there were eleven differences. Of which, five resulted in aa charge changes (Table 4), and five occurred in NS5 at aa residue 2626 (Phe → Ile), 2762 (Ile → Th), 2785 (Tyr → His), 2826 (Leu → Ile), and 2935 (Asp → Glu). NS5 protein has both methyltransferase and viral RNA-dependent RNA polymerase activities essential for viral replication. As shown in Fig. 2(B), comparison of the replication kinetics of the two viruses revealed that ThD2-0433-85 replicated more efficiently in DCs from four of five donors, consistent with the results shown above.

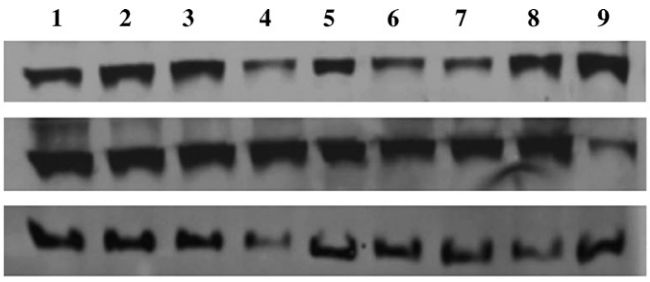
3.4. Comparison of IFNα production and STAT-1 expression in human DCs infected with DF- and DHF-associated viral isolates

The results shown in Fig. 2 demonstrated that the replication of ThD2-0026-88 (DF isolate) was delayed in some experiments raising the possibility that host-induced IFNα differentially regulated viral replication. However, because both ThD2-0026-88 and ThD2-0433-85 elicited similar amount of IFNα from infected DCs (data not shown), it is possible that the observed differences in the replication rates of these viruses reflected differences in their abilities to antagonize the effects of IFNα on the host cell. Because the targeting of STAT-1 by DENV is thought to inhibit IFNα-induced STAT-1 phosphorylation, we used Western Blots to determine total and tyrosine-phosphorylated STAT-1 levels following IFNα treatment of uninfected DCs and DCs infected with DF- and DHF-associated viral isolates including ThD2-0026-88 and ThD2-0433-85 as well as laboratory adapted strains of DENV-2 and -4. As shown in Fig. 3, six out of eight viruses tested suppressed IFNα-induced STAT-1 phosphorylation below the level seen in uninfected DCs treated with

Table 4
Summary of amino acid changes between DHF and DF-associated DENV isolates.

Dengue viruses	Disease severity	Changes of amino acid[a] (gene and position number)													
		PrM	E	NS1	NS2A		NS3			NS5					3′ NTR from stop codon
		130	680	899	1263	1342	1595	1994	2024	2626	2762	2785	2826	2935	13
ThD2-0433/85	DHF	*R*	*R*	I	M	**S**[b]	*A*	I	*Q*	F	*I*	*Y*	L	D	C
ThD2-0026/88	DF	*I*	*Q*	L	I	N	*T*	V	*K*	I	*T*	*H*	I	E	A

[a] Charge changes are italicized. *Hydrophilic-charged amino* acid: H (histidine), D (aspartate), E (glutamate), K (lysine), and R (arginine). *Hydrophobic amino acid*: A (alanine), V (valine), L (leucine), I (isoleucine), M (methionine), and F (phenylalanine). *Hydrophilic neutral*: N (asparagine), Q (glutamine), S (serine), T (theonine), and Y (tyrosine).
[b] Boldface letter was predicted as serine phosphorylation site at score 0.998 (NetPhos 2.0 program).



79.70	86.68	91.79	79.04	77.28	74.94	67.69	90.97	91.62	P-STAT1
112.46	107.75	113.81	130.80	111.65	114.19	105.71	120.73	81.79	Total STAT1
0.71	0.80	0.81	0.60	0.69	0.66	0.64	0.75	1.12	P-STAT1/Total STAT1

Fig. 3. Effect of DF- and DHF-associated virus isolates on IFNα mediated STAT-1 expression in human DCs cultures. Human DCs (2×10^6/ml) were infected with DF and DHF-associated viral isolates at an MOI of 1.0. Twenty-four hours post-infection, infected DCs were treated with 1000 U/ml IFNα for 1 h. Cells were lysed with lysis buffer and supernatants collected by centrifugation. Supernatants from each sample were loaded onto SDS-PAGE gel and immunoblotted to detect phosphorylated and total STAT-1 and h-GAPDH using anti-phospho-STAT-1, anti-STAT-1 (Cell Signalling Technologies), anti-hGAPDH antibodies (Chemicon International Inc., Temecula, CA). The blot shown represents one of two independent experiments. The intensities of P-STAT-1, total-STAT-1, or ratio of P-STAT-1 to total STAT-1 protein expression relative to intensities of hGAPDH are shown. Lane 1: DENV-2 (S16803); Lane 2: ThD2-0026-88; Lane 3: ThD2-0433-85; Lane 4: DENV-4; Lane 5: ThD4-0017-97; Lane 6: ThD4-0734-00; Lane 7: ThD4-0476-97; Lane 8: ThD4-0485-01 and Lane 9: Mock.

IFNα. However, the ability of the viruses to inhibit STAT-1 phosphorylation did not appear to be associated with disease severity. Surprisingly, DENV infection up-regulated total STAT-1 expression in DC cells, a finding not previously reported, and which suggests that virus-mediated suppression of total STAT-1 conversion to functional STAT-1 may have occurred.

4. Discussion

The pathogenesis of DENV infection is still not well understood and likely to be mediated by both host and viral factors. Several hypotheses have been proposed including effects at the host level such as antibody-dependent enhancement (ADE) of infection (Halstead, 1988; Halstead et al., 1970; Kliks et al., 1989) and at the viral level such as inherent differences in virulence among DENV strains (Rosen, 1986). Differences in virulence between naturally circulating virus strains have been reported (Rico-Hesse et al., 1997), and studies report molecular changes in the DENV genome that appear to correlate with disease severity (Leitmeyer et al., 1999; Mangada and Igarashi, 1997; Pandey and Igarashi, 2000). In the present study, we compared DENV strains isolated from patients with different disease outcomes for their binding to host cells, their replication kinetics, and their ability to antagonizing the effect of IFNα on the host. Since most DENVs do not replicate to high titers in the available host cell, this is particularly true of wild-type isolates that have not been previously adapted by serial passage. Our primary intention for this work is to characterize "near wild type" DENV isolates which maintain a high degree of unaltered genetic integrity and diversity. We, therefore, cultivated our DENV isolates with low *in vitro* passages to avoid as much potential selective pressure as possible. Due to limitations in virus titer for some strains and in number of human DCs required for these infections, we were unable to include all the isolated viral strains in the replication kinetic and their ability to antagonizing the effect of IFNα on the host cell experiments. Only those viruses with high PFU/ml were tested.

In this study we found that DENV isolates from patients with more severe disease tended to replicate more rapidly and thus to a higher titer in DCs than viruses from patients with less severe disease. This result is consistent with clinical observations, which show an association between disease severity and serum viremia levels (Libraty et al., 2002; Vaughn et al., 2000), suggesting that the amount of virus replication is proportional to the degree of pathogenesis. In our experiments in DCs we did not attempt to measure the effect of sensitizing antibodies, i.e., on ADE of viral replication mediated through Fc-receptors, on virus yield, nor could we predict what role the host's cell-mediated immune responses might have played in pathogenesis. Arguably, these are very important factors in determining disease outcome. We were also unable to directly demonstrate specific amino acid or nucleotide differences with disease outcome and more studies are required to determine which differences that we observed between DF and DHF isolates are important in pathogenesis.

The ability of DENV to bind to host cell receptors is an important factor contributing to the permissiveness of target cells to infection (Wang et al., 1999). However, it is unknown whether differences in receptor binding *in vivo* play a significant role in disease severity. We examined the ability of several DENV isolates from serotypes 1–4 circulating in Thailand during 1981–2001 to bind DC-SIGN expressed on transfected Raji cells but did not find significant differences in the binding abilities between DF and DHF-associated strains or a correlation with strain dominance at the time of sampling. Our results suggest that other steps in viral replication may have more profound effects on viral virulence, pathogenesis and disease outcome. Thus, successful establishment of DENV infection requires critical steps beyond viral attachment such as internalization via fusion of viral and cellular endosomal membranes, RNA replication, and assembly and maturation of progeny virus.

Following DENV infection several antiviral mechanisms are induced, among which IFNα responses are mediated via the IFNα signaling pathway (Muller et al., 1994; Navarro-Sanchez et al., 2005). Innate cellular immune mechanisms mediated by IFNα are arguably the most immediate challenge to virus survival and many pathogenic viruses encode proteins to counter the IFN response (Co et al., 2007; Heim et al., 1999; Wu et al., 2007). The effectiveness of these mechanisms may play a major role in determining the pathogenic outcome of an infection. Previous studies have shown that virus derived from a DENV-2 infectious cDNA clone (Munoz-Jordan et al., 2003) and laboratory adapted strains of DENV-2 upon infection of target cells (Ho et al., 2005) block the IFNα signaling pathway by interfering with the activation of STAT proteins. Results suggesting that NS2A, NS4A, and NS4B block IFN signaling by reducing STAT activation (Ho et al., 2005; Munoz-Jordan et al., 2003) were later confirmed using cell lines stably transfected with self-replicating DENV replicons that express all the DENV non-structural proteins (Jones et al., 2005). Other positive-stranded RNA viruses such as HCV were also found to encode IFN-signaling inhibitors (Heim et al., 1999). The results of our study are consis-

tent with previous work demonstrating DENV-mediated reduction of IFNα-induced STAT-1 activation (Ho et al., 2005; Jones et al., 2005; Munoz-Jordan et al., 2003; Samuel, 2001). Compared with other investigators we were able to examine a larger number of DENV isolates associated with a range of disease severity on STAT-1 activation by IFNα. The viruses that we studied, with the exception of two isolates, which did not elicit inhibition effects, varied in their ability to inhibit IFNα-induced STAT-1 phosphorylation, however, we observed no significant difference between isolates associated with DF and those associated with DHF. Our results also showed that all the DENV isolates examined elicited higher levels of total STAT-1 expression, a finding not been previously reported. The increased levels of total STAT-1 may represent the accumulation of newly synthesized protein caused by DENV inhibition of upstream molecules in the JAK/STAT pathway. Ho et al. (2005) reported that DENV suppresses STAT-1 phosphorylation by targeting and inhibiting the activation of Tyk2, an upstream molecule that is required for STAT-1 activation.

In summary, our data show that DENV isolates associated with more severe disease (and thus potentially more pathogenic) replicated to higher titers in human DCs than that with less pathogenic isolates, but that the differential replication rates were independent of cell-binding capacity or interference with the IFNα signaling pathway. Future work will be aimed at determining which genetic changes are responsible for the observed differences in viral replication.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virusres.2009.05.016.

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