DETECTION OF DENGUE VIRUS IN PLATELETS ISOLATED FROM DENGUE PATIENTS

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Abstract. Though thrombocytopenia or dysfunction of platelets is common in dengue virus infection, the role of platelets has not been established. We enrolled 33 hospitalized children with serologically confirmed dengue virus infection. Blood specimens were collected during hospitalization. Platelets and plasma were isolated from the whole blood. Detection of dengue virus in plasma and platelets was carried out by RT-PCR with primers that can differentiate different dengue serotypes simultaneously, and by electron transmission microscopy (EM). Dengue viral RNA was detected in the platelets and plasma by conventional RT-PCR. A significantly higher percentage of dengue viral RNA was detected in platelets than in plasma (p=0.03). Platelets isolated 5 days after onset of fever were most likely positive for viral RNA. Concurrent infection or co-circulation with multiple dengue serotypes was observed in 12% of patients. Infrequently, negative-stranded dengue viral RNA was detected in platelets and in plasma. Importantly, EM confirmed the presence of dengue viral-like particles inside platelets prepared from dengue patients. Our findings suggest the presence of dengue virus in platelets may be associated with the dysfunction of platelets observed in dengue patients.

INTRODUCTION

Infection with dengue virus (DENV) is considered a major public heath issue internationally (Mackenzie *et al*, 2004). Dengue virus infection is the most important mosquito borne human disease in terms of morbility and mortality in urban tropical areas. Changing climate, such as global warming and the effects of El Nino, unplanned urbanization, increased international travel, and the lack of effective vector-control programs are predominant causes of the increased threat of dengue infection (Barclay, 2008). The National Institute of Allergy and Infectious Diseases (NIAID) has listed dengue virus as a Category A priority bio-threat pathogen (NIAID, 2005). Over 100 million people living in tropical and subtropical areas are at risk of infection with the dengue

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virus. An estimated 50 million cases of dengue infection are documented globally per year. The death rate due to dengue infection is approximately 2 to 5%, and occurs predominantly in children under 15 years of age (WHO, 2004). The recent outbreak of dengue infection in Brazil highlights the potential for the virus spread to the Americas (Morens and Fauci, 2007). Dengue infection is a potential threat to public health in the US. Currently, there is no known vaccine or effective chemotherapeutic treatment.

Dengue virus is a single-stranded, positive sense RNA virus whose genome is 11 kb in length. The virus is spherical in shape, 40-50 nm in diameter. A single large protein is translated from the positive RNA and is digested into viral components by viral or host proteases. There are four serologically distinct but geneticcally related dengue viruses: DENV-1, DENV-2, DENV-3, and DENV-4. The *Aedes aegypti* mosquito is responsible for most of the transmission (WHO, 2004).

The spectrum of dengue disease includes asymptomatic cases, dengue fever (DF), dengue hemorrhagic fever (DHF), and dengue shock syndrome (DSS) (Anderson *et al*, 2007). DENV infections can be life threatening, usually when caused by secondary infection with heterologous virus serotype (WHO, 2004). The incubation period of DENV infection varies from 5 to 10 days. Viremia occurs roughly 2 days prior to clinical manifestations and lasts for 5-6 days (Halstead, 2007).

One of the clinical hallmarks in dengue virus infection is thrombocytopenia or dysfunction of platelets. There are three mechanisms that can lead to thrombocytopenia: decreased production, increased consumption, or immune-complex lysis (Srichaikul and Nimmannitya, 2000; Schexneider and Reedy, 2005; Oishi *et al*, 2007). However, the precise mechanism for the development of thrombocytopenia or dysfunction of platelets in dengue infection remains elusive.

Platelets are anuclear cells derived from megakaryocytes. Although the size of platelets is small, platelets carry out biological functions, including protein synthesis and protein modification; they possess receptors on their surface for signal transduction. Importantly, several platelet surface receptors linked to the entry of the dengue virus, such as DC-SIGN or enhancement of the receptor FcγII have been found (Michelson, 2007). With the era of microarray and proteomic technology, the understanding of platelet function has expanded in recent years, including the understanding that platelets act as immune cells (von Hundelshausen and Weber, 2007), cross-talking with lymphocytes (Li, 2008) and being actively involved in shaping the immune response upon encountering infectious agents (Elzey *et al*, 2003).

In dengue infection, studies have found viral antigen on the surface of platelets, immune-complex containing platelets on skin biopsy and an association between dengue virus and platelets *in vitro* (Boonpucknavig *et al*, 1979a,b; Saito *et al*, 2004; Oishi *et al*, 2007). However, no active dengue virus products have been demonstrated in platelets from infected patients. The exact role of platelets in the pathogenesis of dengue virus infection and its association with thrombocytopenia and dysfunction of platelets is still unknown. We enrolled 33 clinically and serologically confirmed dengue fever patients into this study to investigate the relationship between platelets and dengue virus, and the potential association with clinical outcome.

MATERIALS AND METHODS

Patients and samples

The patients enrolled in the study were children hospitalized at Siriraj Hospital,

Mahidol University, Bangkok, Thailand with a clinical diagnosis of DF or DHF according to WHO criteria from November 2006 to September 2007. Two to 3 milliliters of blood was collected in an EDTA tube (Vacutainer, Becton Dickinson, Franklin Lakes, NJ) from each patient following hospital admission after informed written consent was given in accordance with a protocol approved by the Siriraj Hospital Ethics Committee. Thirty-three patients with serological confirmation of acute dengue infection were studied.

Isolation of human platelets and plasma

Human platelets and plasma were isolated from collected blood using OptiPrep (Axis-Shield, Monroe, NC). Briefly, 5 volumes of OptiPrep were diluted with 22 volumes of 0.85% (w/v) NaCl, 1 mM EDTA, 20 mM Herpes-NaOH, pH 7.4 to produce a 1.063 g/ml solution. In a 15-ml centrifuge tube, 2-3 ml of blood was layered over 5 ml of the 1.063 g/ml solution and centrifuged at 350*g* for 15 minutes at 22ºC in a swinging bucket rotor with no break during deceleration. The clear plasma layer was removed and thereafter the broad turbid band containing platelets, which extended into the density barrier from just above the interface, was harvested. The collected plasma and platelets were aliquoted, and subjected to RNA extraction and EM study.

RT-PCR

RNA was extracted from 140 μl of human platelets and plasma using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction and resuspended in 12 μl of DEPC-treated H_2O. Five μl of total RNA was subjected to reverse transcription (RT) with primer DEUR (5'-GCT GTG TCA CCC AGA ATG GCC AT-3') and multiplex nested PCR with four primer pairs specific for the E region of each dengue serotype to detect positive stranded dengue viral RNA according to a previous report with minor modifications (Yenchitsomanus *et al*, 1996). The expected sizes of the nested PCR products for DENV-1, DENV-2, DENV-3, and DENV-4 were 504 bp, 346 bp, 196 bp, and 143 bp, respectively. For detection of negative stranded dengue viral RNA, 5 μl of the extracted RNA was utilized for RT with primer Tag F (5' CGG TCA TGG TGG CGA ATA AGG TTA GAG GAG ACC CCT CCC-3') and cDNA was purified with a PCR purification kit (Qiagen, Hilden, Germany) and resuspended in 30 μl of 10 mM Tris-HCl, pH 8.0. PCR was performed with sense and anti-sense primers specific for the 3'UTR region, Tag, 5' CGG TCA TGG TGG CGA ATA A-3', and R1, 5' GGC GTT CTG TGC CTG GA, respectively with Immolase DNA buffer according to manufacture's protocol (Invitrogen). The Primary amplicons were then subjected to semi-nested PCR with a primer pair, Tag and R2 (5' GAG ACA GCA GGA TCT CTG GTC T-3').

Electron microscopy for detection of dengue viral-like particles in platelets

Freshly isolated platelets were fixed with 4% glutaraldehyde in PBS overnight at 4ºC and washed once with PBS followed by a phosphate buffer of pH 7.3 three times (3 minutes each). The platelets were then incubated with 2% phosphate-buffered osmium tetroxide for 45 minutes, washed with distilled water, and stained with 2% aqueous uranyl acetate for 30 minutes at RT. The sample was dehydrated at RT by sequential incubation (2-3 times for each step) with 70% and 80% ethanol for 1.5 minutes as well as 90% and 95% absolute ethanol and propylene oxide for 3 minutes. Thereafter, the sample was infiltrated with a mixture of propylene oxide and epoxy resin (50:50) at 37ºC for 30 minutes followed by an epoxy resin mixture alone for 2 hours at 37ºC. The sample was embedded in a polypropylene capsule and allowed to polymerize at 70ºC overnight. Images were captured with

Hitachi Transmission Electron Microscope.

RESULTS

Thrombocytopenia or dysfunction of platelets in DHF/DSS patients normally occurs after the onset of fever. Our patients were predominantly enrolled 4 to 7 days after the onset of fever (Fig 1A). Of the 33 patients enrolled, the average age was 8 years (range 2-14) and the male to female ratio was 2:1 (Fig 1B). A summary of the clinical diagnoses is shown in Table 1. The diagnosis was DHF in 27 patients and DF in 6 patients.

RNA was isolated from purified platelets and plasma and RT-PCR was performed as described above. The patients' RT-PCR profiles are shown in Fig 2. The cumulative results are summarized in Table 1. A higher percentage of dengue viral RNA was detected in isolated platelets (52%, 17/33) compared to plasma (30%, 10/33). A negative stranded RNA was observed in some purified platelets and plasma, 5/33 and 2/33 (Table 1), respectively. The results suggest that active viral activities, to a certain extent, may occur within the platelets, or these are heritage products from parental cells. Co-circulation of multiple serotypes of dengue virus was observed in 12% of the enrolled patients (Table 1). The diagnostic outcomes of dengue virus infection are shown in Table 1. Varying degrees of DHF severity were observed in the enrolled patients.

A single positive stranded RT-PCR was performed on each patient's blood sample and tabulated according to the day of onset of fever (Table 2). Dengue viral RNA was detected in platelets from cases enrolled 5 days after the onset of fever at a significantly greater rate compared to plasma (p=0.03). Among those with dengue virus detected by RT-PCR, dengue RNA was isolated in platelets significantly more often than in plasma (p=0.03, Table 2). The relationship between

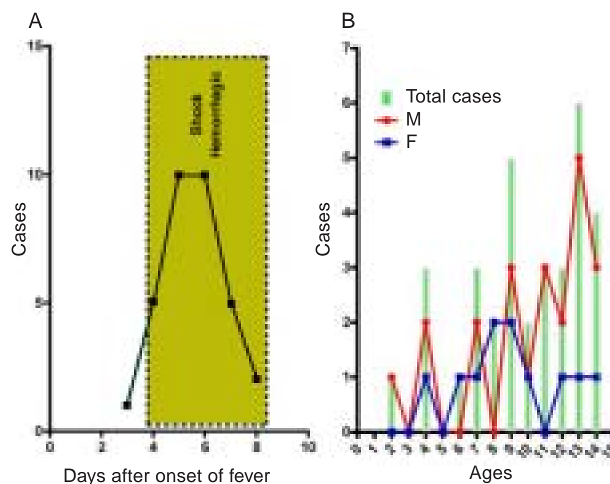

Fig 1–Dengue virus infection profile. (A) Enrolled cases were predominantly within the periods of dengue hemorrhagic fever or dengue shock syndrome. (B) Distribution by sex and age of enrolled patients. IgM1 and IgG1, primary infection; IgM2 and IgG2, secondary infection.

the number of RNA positive cases and the daily average platelet count is found in Table 2. The nadir platelet count was observed on the 5th day after the onset of fever.

Electron microscopy was applied to investigate the relationship between dengue virus and platelets. Frequently, a cluster of dengue viral-like particles was observed inside a vacuole or an endosomal vesicle in platelets isolated from dengue patients (Fig 3, A and B). Occasionally, a single isolated dengue viral-like particle was observed inside the platelet (Fig 3C).

DISCUSSION

Thrombocytopenia and dysfunction of platelets are key features in dengue infections. This normally occurs 4 to 7 days after the onset of fever (Srichaikul and Nimmannitya, 2000). In order to better understand this

Table 1
Summary of clinical diagnosis and dengue viral RNA in platelets and plasma from enrolled patients.

Subject	Age	Gender	DOF[a]	Diagnosis[b]	Positive stranded RNA[c]		Negative stranded RNA[d]	
					Platelets	Plasma	Platelets	Plasma
1	7	F	4	DF	-	-		
2	13	M	7	DHF#	Denv-2[e],3	Denv-2,3		
3	14	F	5	DHF2	Denv-2[e]	-	+[e]	
4	8	F	6	DF	Denv-1	Denv-1		
5	13	F	8	DHF2	Denv-4	-		
6	12	F	6	DHF1	Denv-4	-		
7	8	F	7	DHF1	Denv-2,3	Denv-2[e],3	+	+[e]
8	4	M	7	DHF1	-	-		
9	13	M	5	DHF2	Denv-2[e],3	-		
10	9	M	5	DHF1	Denv-4	-		
11	2	M	5	DHF#	Denv-1	Denv-1	+[e]	
12	10	F	6	DHF#	Denv-3	Denv-3		
13	4	F	7	DHF3	-	-		
14	13	M	5	DHF2	Denv-3	-		
15	9	M	6	DHF2	-	-		
16	14	M	5	DHF#	Denv-2[e]	-		
17	7	M	4	DHF2	-	-		
18	13	M	3	DF	-	-		
19	6	F	4	DHF2	Denv-3[e]	-		
20	4	M	7	DHF3	-	-		
21	11	M	4	DHF4	-	Denv-1[e]		
22	12	M	4	DHF1	-	-		
23	11	M	5	DHF2	-	-		
24	9	F	6	DHF3	-	-		
25	11	M	6	DHF1	Denv-3	Denv-3	+[e]	+
26	9	M	5	DF	-	-		
27	14	M	6	DF	-	-		
28	10	M	5	DHF1	Denv-3	Denv-2,3[e]	+[e]	
29	14	M	8	DHF2	-	Denv-4[e]		
30	13	M	6	DHF3	Denv-3	-		
31	7	M	5	DF	Denv-3	Denv-3		
32	12	M	6	DHF3	-	-		
33	9	F	6	DHF2	-	-		
					17/33	10/33	5/33	2/33

[a]DOF, Day after onset of fever based on patient's recollection of the first day of fever, specimens were collected.
[b]Clinical diagnosis was made according to the WHO defined criteria; however, different grades of DHF were not specified in some patients (#).
[c]Dengue positive stranded viral RNA was assayed by conventional RT-PCR, which can simultaneously differentiate dengue serotype.
[d]Dengue negative stranded viral RNA was assayed as described in Methods. "+" indicates detection of negative stranded RNA.
[e]Nucleotide sequencing was performed to verify the serotype.

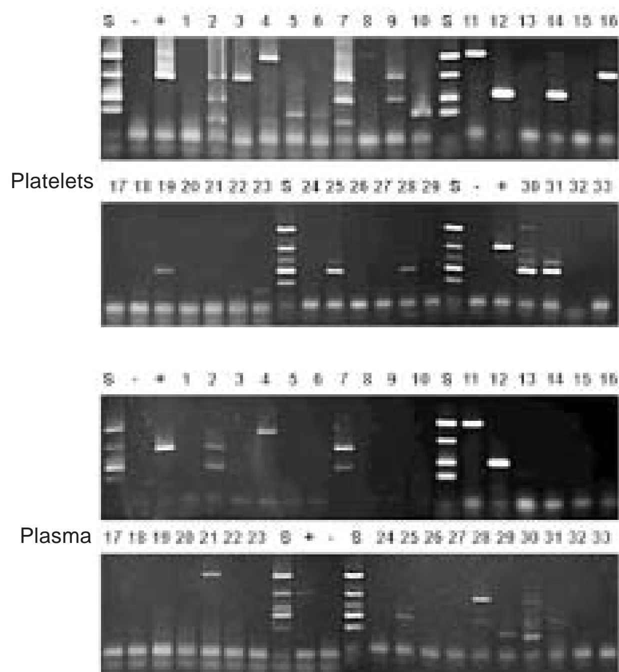

Fig 2–Dengue viral RNA in platelets and plasma. Dengue viral RNA isolation and performance of RT-PCR are described in the Methods section of this paper. The RT-PCR results are shown. A higher percentage of dengue viral RNA was seen in platelets than plasma. S, standard markers of dengue serotype 1, 2, 3, and 4; -, negative control; +, positive control.

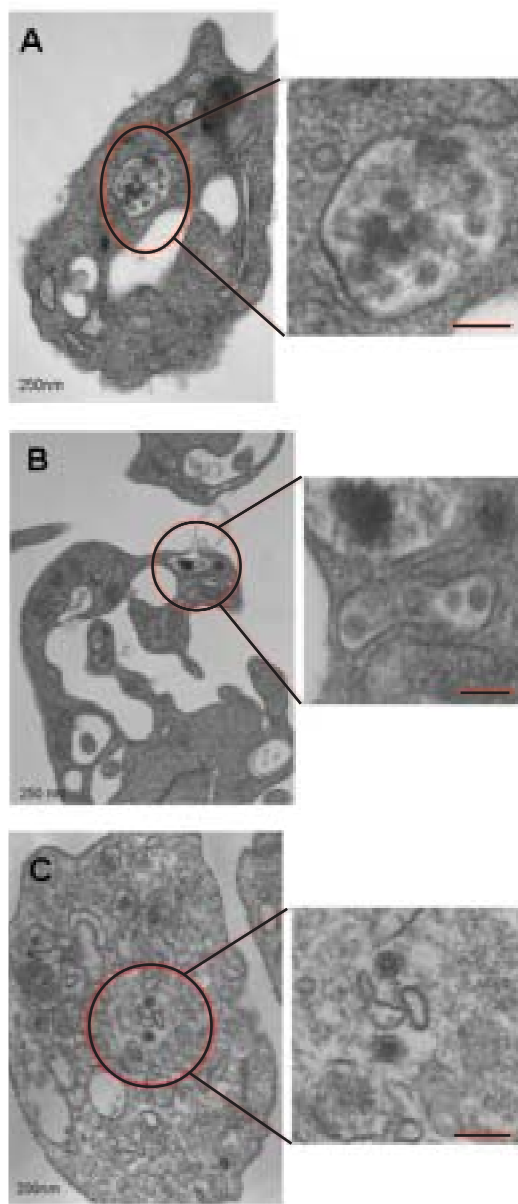

Fig 3–Detection of dengue-like viral particles in platelets with EM. Preparation of isolated platelets for EM study is described in the Methods section of this paper. EM images from three patients are shown. Clustering (A and B) or single isolated (C) dengue-like viral particles were observed inside the platelets. Platelets containing dengue-like viral particles appeared to be activated. Bar in inset: 100 nm.

clinical phenomenon, we examined the platelets in confirmed dengue infected patients and demonstrated that dengue virus was observed inside platelets raising the possibility that platelets may have harvested dengue virus.

It has been known for quite some time that platelets are able to engulf foreign particles, such as latex beads, bacteria and viruses (White and Clawson, 1982; Zucker-Franklin, 1994). With the advent of gene and proteomic array technology, numerous important immune related markers have been

Table 2
Summary of daily cases based on dengue viral positive stranded RT-PCR assays in platelets and plasma.

DOF	3	4	5	6	7	8	Total
RT-PCR positive cases[a]	0 (1)	2 (5)	8 (10)	5 (10)	2 (5)	2 (2)	19 (33)
RT-PCR platelets[b]	0	1	8	5	2	1	17
RT-PCR plasma[c]	0	1	3	3	2	1	10
P-value[d]			0.0256				0.0293
Ave. platelets[e]	108±0.00	101±67	69±38	71±49	85±73	74±15	

DOF, Day after onset of fever.
[a]Daily RT-PCR positive cases in platelets and/or plasma. Number in parenthesis indicates daily available specimens.
[b]Distribution of RT-PCR positive cases in platelets.
[c]Distribution of RT-PCR positive cases in plasma.
[d]*P*-value was analyzed by two-sided Fisher's exact test. Significant is defined as when $p<0.5$.
[e]Average daily platelet counts (x10^3 cells/μl).

found in or on the surface of platelets, such as DC-SIGN, CD40 ligand, complement receptor, blood group antigens, and IgG FcγII receptor, a unique receptor found only in human platelets (Lindemann and Gawaz, 2007; Michelson, 2007). It has been documented that platelets can ingest blood-borne viruses and bacteria (Zucker-Franklin, 1994; Youssefian *et al*, 2002) and associate or adhere to immune cells, in particular, monocytes and macrophages (Michelson, 2007). Thus, the role of platelets has been extended to include immune function, bridging the innate and adaptive immune system (Elzey *et al*, 2003; Lindemann and Gawaz, 2007; von Hundelshausen and Weber, 2007; Li, 2008). However, the actual role of thrombocytopenia and dysfunction of platelets in dengue illness remains largely unknown.

With EM study, we demonstrated that dengue viral-like particles clustered inside an endosomal vesicle structure or phagosome-like vesicles, which is similar in appearance to what has been observed in other cells infected with dengue virus (Rahman *et al*, 1998; Kwan *et al*, 2008). Although negative stranded RNA was detected (Table 1) in platelets isolated from some dengue infected patients, we were also able to recover dengue virus by co-cultivation of the platelets isolated from one of these patients with Vero cells (data not shown). This is consistent with a previous work reporting that dengue virus can be isolated from platelets of dengue patients infrequently (Scott, 1978). In addition, our results support a recent study by Saito *et al* (2004), in which dengue viral RNA was detected in platelets isolated from secondary dengue virus infected patients. The observation of low recovery of infectious dengue virus in platelets is similar to dengue virus infected macrophages (Kwan *et al*, 2008), suggesting the majority of dengue virus was in the phagosomes of platelets. Whether the presence of dengue virus in platelets leads to a platelet dysfunction or thrombocytopenia observed in dengue patients requires further investigation.

In our study, most patients enrolled 4-7 days after the onset of fever, had detectable

dengue viral RNA in their platelets at a significantly higher level than in the plasma during the same period. However, we did observe dengue viral RNA in the plasma of two patients, but not in their platelets. Their specimens were collected on different days, 4 and 8 days after the onset of fever. This observation may be due to 1) the timing of the specimen collection, since fluctuations of dengue viral load were noticed in individuals' daily specimen and 2) the presence of microparticles in plasma, presumably derived from platelets. Interestingly, the majority of microparticles circulating in blood are from activated platelets (Horstman and Ahn, 1999). However, we were unable to evaluate whether microparticles derived from other immunes cells may have contributed to our observations.

Even with the limited number of patients, we observed all 4 dengue serotypes. This observation is consistent with studies performed in endemic regions. Co-infection with multiple dengue serotypes simultaneously does not increase the severity of the disease (Gubler, 1998; Lorono-Pino *et al*, 1999; Chinnawirotpisan *et al*, 2008; Gupta *et al*, 2008). We found dengue virus easily detectable in platelets during the latter part of the illness, the period during which a lower percentage of the virus can be recovered or detected in the plasma (Klungthong *et al*, 2007). This may explain why the detection of dengue virus in whole blood is much more sensitive than in plasma or serum (Klungthong *et al*, 2007). Interestingly, we found the nadir platelet count correlated with the greatest number dengue viral RNA particles detected in the platelets 5 - 6 days after the onset of fever. Only about 60% (19/33, combined platelets and plasma) of cases with dengue viral RNA had positive serological results for dengue virus. The rate of RNA detection in our study is similar to that reported by others (Klungthong *et al*, 2007; Kumarasamy *et al*, 2007). Whether the dengue virus infected platelets are part of the host defense role, or do they enhance immune-complex mediated lysis and IgG-FcγII mediated phagocytosis by macrophage or dendritic cells, resulting in the loss of function in platelets, is unclear. We could not rule out the possibility that these dengue viral RNA positive platelets were directly derived from megakaryocytes or their progenitor cells.

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