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BRIEF REPORT

Dengue Virus Entry and Replication Does Not Lead to Productive Infection in Platelets

Meenakshi Kar,[1] Mohit Singla,[2] Anmol Chandele,[3] Sushil K. Kabra,[2] Rakesh Lodha,[2] and Guruprasad R. Medigeshi,[1a]

[1]Vaccine and Infectious Disease Research Center, Translational Health Science and Technology Institute, Faridabad, Haryana; [2]Department of Pediatrics, All India Institute of Medical Sciences, New Delhi; and [3]ICGEB-Emory Vaccine Center, International Center for Genetic Engineering and Biotechnology, Aruna Asaf Ali Marg, New Delhi, India

Thrombocytopenia is a characteristic feature during the acute phase of dengue infection and has been found to associate with vascular leakage in severe dengue. Although dengue antigens have been observed in platelets, there is no strong evidence to suggest a direct infection of platelets by dengue virus as a contributing factor for thrombocytopenia. We show that dengue virus can enter platelets but replicate viral ribonucleic acid to a minimal extent and, therefore, cannot produce infectious virus. Dengue antigen was undetectable in platelets isolated from dengue patients; however, we observed an increase in $CD14^+CD16^+$ monocyte-platelet complexes, suggesting a mechanism for platelet clearance.

Keywords. dengue virus; monocytes; platelets.

The number of dengue incidences has more than doubled over the last 2 decades, currently accounting for approximately 60 million cases annually [1]. Severe dengue is one of the leading causes of hospitalization and death in the pediatric population, and the highest dengue mortality occurred in southeast Asia [1]. Dengue clinical features span from mild dengue fever to severe shock, which involves plasma leakage and hemorrhage. The course of the disease often coincides with a severe drop in platelet counts, which correlates with vascular leakage [2]. Studies involving dengue patients and animal models have found several associations with thrombocytopenia, which include the following: altered hematopoiesis during acute phase, complement activation and platelet destruction, platelet activation and degranulation, and impaired platelet functions [2–6]. Other studies have proposed direct infection of platelets based on detection of dengue antigen or dengue virus (DENV) ribonucleic acid (RNA) in platelets from dengue patients or in vitro infection of platelets by DENV; however, there is no clear demonstration of productive dengue infection in platelets by these studies [7, 8]. In the current study, we elucidate whether DENV productively infects platelets by in vitro infection of platelets derived from healthy adults, and we examine platelet infection by DENV in infected patients. We find that DENV enters platelets, but this does not result in a productive infection. However, platelets activated by DENV form complexes with monocytes, primarily the $CD14^+CD16^+$ double-positive monocytes, which may result in platelet apoptosis and lead to thrombocytopenia. Our results provide insights into one of the possible mechanisms of thrombocytopenia in dengue infections.

METHODS

Screening and Enrollment of Dengue Patients

The study was approved by the institutional ethics committees of the 3 participating institutes. All patients in this study were enrolled between September 2015 and Novermber 2015 at the Department of Pediatrics, All India Institute of Medical Sciences (AIIMS), New Delhi. Four milliliters of blood samples were collected once at the time of enrollment after testing positive in a NS1 antigen test. Patient screening, enrollment, analysis of clinical features, and classification of disease severity were carried out as described previously [9]. In brief, children aged 4–14 years and presenting to AIIMS with symptoms suggestive of dengue were screened using a rapid test for dengue NS1 antigen and differential detection of immunoglobulin (Ig)M and IgG antibodies to DENV in serum/plasma (J. Mitra and Co. Pvt. Ltd, New Delhi). Patients who tested positive for either NS1 or IgM were informed and were asked to participate in the study. Disease severity was assigned at the time of enrollment and was based on the latest World Health Organization (WHO) grading and divided into 3 groups: severe dengue (SD), dengue with warning signs (DW), and dengue illness (DI). The admitted patients were managed according to standard protocols of the admitting physician, whereas the outpatients were followed up at alternate-day intervals until recovery, and patients were free to visit earlier if necessary. Dengue serotyping, viremia estimation, and dengue antibody capture enzyme-linked immunosorbent assay (ELISA) to determine primary and secondary infection were performed exactly as described previously [9].

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Correspondence: G. R. Medigeshi, PhD, Associate Professor, Translational Health Science and Technology Institute, NCR-Biotech Science Cluster, P.O. Box #4, Faridabad-Gurgaon Highway, Faridabad 121001, India (gmedigeshi@thsti.res.in).

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Isolation of Peripheral Blood Mononuclear Cells and Platelets From Dengue Virus Patients and Flow Cytometry

Peripheral blood mononuclear cell (PBMC) isolation and staining for DENV antigen and PBMC subsets, gating of cell populations, bulk sorting of PBMCs, and detection of viral RNA in PBMC subsets were performed as described previously [9]. Platelets (0.25×10^6) present in the platelet-rich plasma (PRP) fraction obtained in PBMC isolation were stained for DENV antigen as described below. Platelet-leukocyte complexes present in PBMC fraction were determined by staining with the following antibodies: CD3-APC-Cy7, CD14-PerCP-Cy5.5, CD16-PE, CD19-FITC, CD41a-PE-Cy7, Live/Dead BV450, Dengue-E-APC. Samples were acquired in FACS-Canto (Becton Dickinson) with voltage settings on a logarithmic scale.

Platelet Isolation From Healthy Volunteers and In Vitro Infection With Dengue Virus

Platelets were isolated as described previously with minor modifications [10]. In brief, 5 mL blood was collected from healthy adult volunteers (age 23–25 years, 2 female, 2 male) in acid citrate dextrose buffer (39 mM citric acid, 75 mM sodium citrate and 135 mM dextrose) in a 9:1 ratio. Platelet-rich plasma was collected from whole blood after centrifugation at 50 × *g* for 15 minutes at room temperature without brakes. Platelets were pelleted by centrifugation of PRP at 1000 × *g* for 10 minutes at room temperature. Platelet pellet was washed and resuspended in HEPES Tyrode's buffer containing 1 µM prostaglandin E1. Platelet purity was assessed by flow cytometry using CD41a-PE-Cy7 and CD45-PerCP-Cy5.5 antibodies. Platelet viability was assessed by staining with 0.5 µM Calcein AM dye (Thermo Fisher Scientific) for 15 minutes in the dark at room temperature before adding antibodies. For DENV-2 (GenBank accession no. KJ918750.1) infection, 3×10^5 platelets resuspended in serum-free Roswell Park Memorial Institute (RPMI) medium were plated and infected with 10 plaque-forming units/cell for 2 hours at 37°C. Unbound virus was removed by washing once with phosphate-buffered saline and resuspended in fresh serum-free medium and continued incubation at 37°C with 5% CO_2 for indicated periods. At each time point, platelets were processed for staining with CD41a-PE-Cy7 and anti-DENV-E-APC antibodies, and samples were acquired in FACS-Canto (Becton Dickinson) with voltage settings on a logarithmic scale.

Plaque Assays and Real-Time Polymearse Chain Reaction

Culture supernatants at 1, 24, 48, and 72 hours postinfection (hpi) were collected, and viral titers were estimated by plaque assay as described previously [11]. For DENV RNA detection by reverse-transcription polymerase chain reaction (RT-PCR), total RNA was extracted from platelet pellet at the indicated time points using Trizol (Invitrogen), and 200 ng of RNA was used in multiplex TaqMan one-step RT-PCR with DENV primer-probe mix [12] and human actin primer-probe mix (Applied Biosystems). Relative expression was calculated using the comparative threshold cycle (ΔΔCt) method. Positive- and negative-strand RNA was detected as described previously [13].

Statistical Analysis

All data were analyzed by GraphPad Prism software. Statistical significance was assessed by Mann-Whitney *U* test between the 2 groups.

RESULTS

Dengue Infection in Platelets

Direct infection of platelets by DENV leading to platelet activation and death has been suggested to be a possible mechanism for thrombocytopenia. To further clarify the role of DENV infection in platelets, we purified platelets from healthy subjects. Platelet fraction was assessed for viability by staining with Calcein AM, a cell-permeable viability dye, and costaining with platelet marker CD41a. Platelet purity was assessed by the absence of leukocyte marker CD45 (Supplementary Figure S1A–C). Platelets have been shown to undergo apoptosis when cultured at 37°C for prolonged periods [14]. We found that platelet viability was significantly compromised either in RPMI or M199 medium, and approximately 70% of the platelets were nonviable by day 4 in culture, and this number did not change further for the duration of the experiment, which is in agreement with previous studies [8] (Supplementary Figure S2A and S2B). Platelets were infected with 10 MOI of DENV-2, and the percentage of dengue infection in platelets was assessed by plaque assays from the supernatant and by intracellular staining of DENV-E protein in the CD41a-positive population at 24, 48, and 72 hpi using flow cytometry. We were surprised to find that no plaques were obtained by plaque assays, and no dengue-positive cells were detected at any of the time points measured in the experiment by flow cytometry, suggesting that platelets were not productively infected with DENV (Figure 1A). To further verify whether any infectious virus was produced at all in platelets upon DENV infection, we infected platelets as described above; at 1, 24, 48, and 72 hpi, we lysed platelets to release intracellular virus; and viral titers in the lysates were measured by plaque assays. We were not able to detect any infectious virus in platelets, despite the fact that intracellular virus was detectable at 1 hpi, suggesting that the virus was entering platelets but failing to replicate or produce infectious virus (Figure 1B). We next verified whether platelets were permissive for DENV replication by infecting platelets as descrived above and measuring positive- and negative-strand DENV RNA in the platelets at 1 and 24 hpi by RT-PCR. Dengue virus was capable of entering platelets because DENV positive-strand RNA was detected in platelets at 1 hpi (from input virus) and the positive-strand RNA levels increased approximately 10-fold by 24 hpi (Figure 1C). Dengue virus negative-strand RNA was detectable at 24 hpi, clearly indicating that DENV RNA replicates in platelets. The ratio of positive to negative strand was approximately 10:1, similar to that reported in flavivirus infections (Figure 1C).

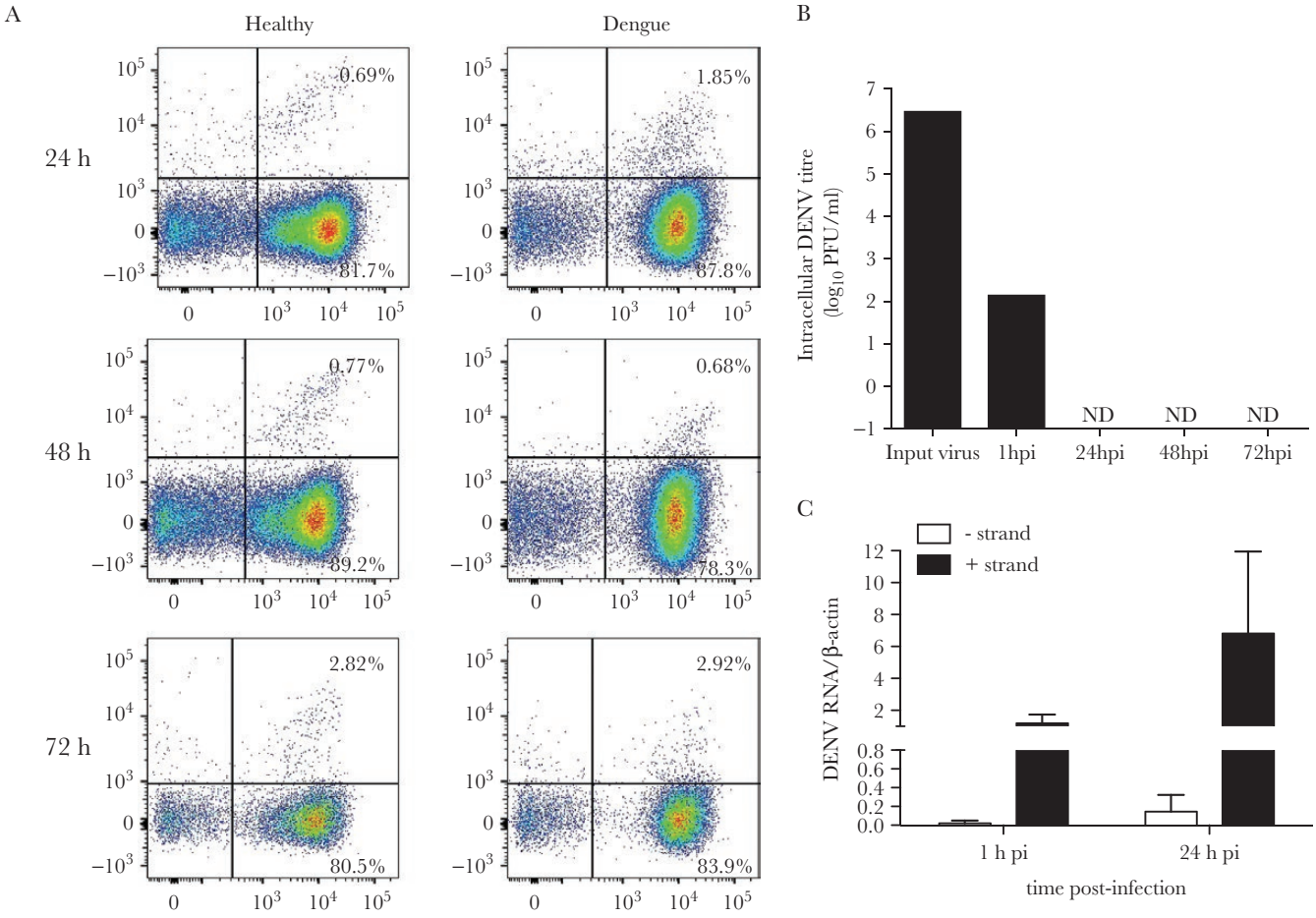

Figure 1. In vitro infection of platelets with dengue virus (DENV)-2 virus. (A) Platelets isolated from healthy adults were infected with 10 multiplicity of infection of DENV-2 and stained for E protein at designated time points. Plots are representative of 2 experiments performed with 2 replicates. (B) Platelets were infected as described above, and at indicated time postinfection platelets were lysed and intracellular virus titer was determined by plaque assays in BHK cells. Bars represent geometric mean values. Data are representative of 2 experiments. (C) Platelets were infected with DENV-2 as described above, and total ribonucleic acid (RNA) was isolated at indicated time points postinfection. Positive- and negative-strand RNA was detected by reverse-transcription polymerase chain reaction. β-actin messenger RNA levels were used for normalization to calculate fold change. Data are combined fold-change values of 2 sets of experiments performed in 3 replicates. Error represents mean with standard deviation. hpi, hours postinfection; ND, not detected; PFU, plaque-forming units.

Platelet Infection in Pediatric Dengue Patients

In a cohort of pediatric patients infected with DENV, we recently showed that monocytes are the major cell types positive for DENV antigen [9]. To further confirm direct platelet infection in DENV patients, we enrolled 23 patients who were NS-1 positive and were within day 5 of onset of clinical symptoms, to capture the active viremic phase. Patients were classified based on 2009 WHO classification, and clinical parameters were determined as described previously [9]. The clinical features of these patients are presented (Supplementary Table S1). Using dengue antibody capture ELISA, we identified 10 patients as seronegative, 7 patients as primary infection, and 6 patients as secondary infections. Similar to the previous study, the majority of the patients were infected with DENV2 (13 of 23) (Supplementary Table S2). We were able to estimate blood viremia in 21 patients: 10 of these cases were mild dengue infection (DI), 7 were DW, and 4 samples were from severe dengue patients (SD). We observed a higher viremia in severe patients compared with DI and DW (Supplementary Figure S3), suggesting a link between viremia and severe disease in these set of patients. We isolated PBMCs from blood samples, and DENV-E positivity in $CD3^+$ (T cells), $CD19^+$ (B cells), $CD14^+$ (monocytes), $CD16^+$ (monocytes and/or natural killer [NK] cells), and $CD41a^+$ (platelets) positive subpopulations was assessed by intracellular staining for DENV-E antigen. We could not detect any significant dengue-positive stain in platelets from any of the patients analyzed in this study (Figure 2A). We found that, in addition to monocytes ($CD14^+$ and $CD14^+16^+$), DENV-E was detected in both $CD19^+$ and $CD16^+$ cells but not in $CD3^+$ cells (Supplementary Figure S4). To further confirm whether the cell types positive for dengue antigen support active dengue replication, we performed bulk sorting of total PBMCs by flow cytometry to purify the 4 major subpopulations, ie, $CD3^+$, $CD19^+$,

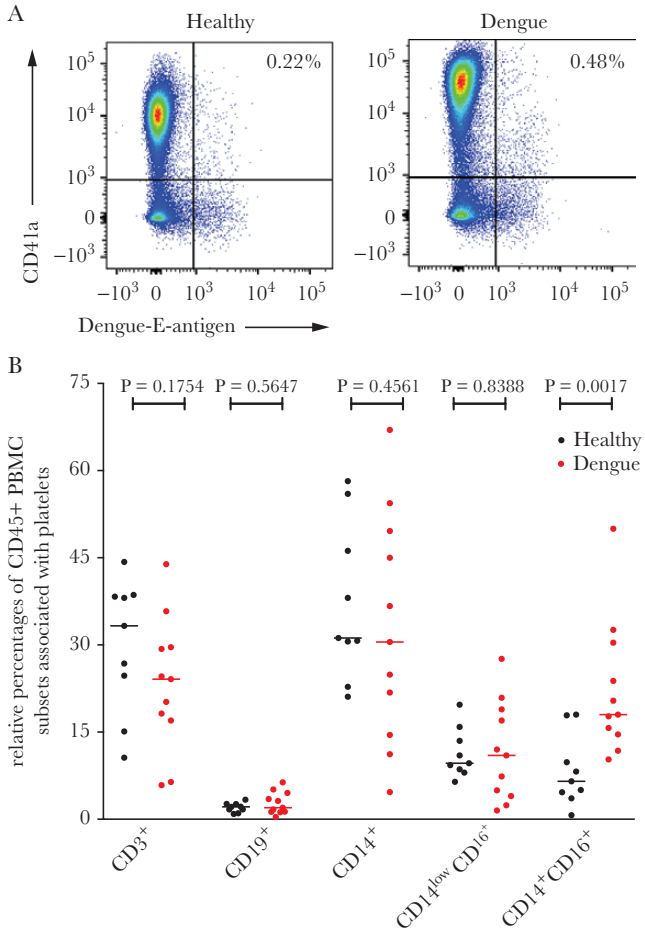

Figure 2. Platelet infection and platelet-leukocyte association in dengue patients. (A) A representative figure of platelets isolated from dengue patients stained with CD41a and anti-DENV-E antibody. (B) $CD41a^+$ platelets were identified in $CD45^+$ population, and the frequencies of indicated subset associated with $CD41a^+$ was estimated relative to total $CD45^+$ cells associated with platelets. N = 9 and N = 11 for healthy controls and dengue patients, respectively. Bar represents median value.

$CD14^+$, and $CD16^+$ cells, at an average purity of >95% and isolated total RNA. A representative purity check of the sorted subpopulation is shown in Supplementary Figure S5. Negative-strand RNA of DENV in these subpopulations was detected by RT-PCR. Of the 12 samples processed for sorting, we detected negative-strand RNA in a total of 6 PBMC samples. All 6 of these samples had blood viremia above 2000 DENV genome equivalents/mL blood, suggesting this as a threshold value for detecting DENV negative strand in PBMCs in our assays (Supplementary Table S3). $CD14^+$ cells from all 6 of these positive samples also showed the presence of negative-strand DENV RNA. $CD19^+$ and $CD16^+$ cells from 4 of the 6 samples showed the presence of negative-strand DENV RNA. None of the 6 samples had negative-strand RNA in $CD3^+$ cells (Supplementary Table S3). Therefore, our results clearly indicate that DENV replicates primarily in B cells, monocytes, and $CD16^+$ cells, which could be intermediate monocytes or NK cells.

Platelet-Leukocyte Complexes in Dengue Patients

Platelets are found to be activated both in dengue patients and upon in vitro infection with DENV [3, 15]. Activated platelets have been shown to bind not only monocytes and neutrophils but also T cells and NK cells [16]. We next examined the platelet-leukocyte complexes in healthy controls and DENV patients by quantitating the $CD3^+$, $CD19^+$, $CD14^+$, $CD14^{low}CD16^+$, and $CD14^+CD16^+$ cells associated with platelets. We observed that frequencies of $CD3^+$, $CD19^+$, $CD14^+$, and $CD14^{low}CD16^+$ cells associated with platelets in DENV patients was similar to healthy controls; however, a significantly higher proportion of platelet-$CD14^+CD16^+$ complexes were found in DENV patients (Figure 2B).

DISCUSSION

We have previously shown an increase in the $CD14^+CD16^+$ cell population at early stages of DENV illness, and these cells were highly positive for DENV antigen within total PBMCs examined [9]. Because a previous report showed that platelets exposed to DENV associated with normal monocytes, compared with DENV-exposed monocytes, in this study, we asked 2 questions: (1) does exposure to DENV result in productive infection in platelets, and (2) do these DENV-exposed or activated platelets form leukocyte-platelet aggregates? Our analysis revealed that DENV enters and replicates in platelets, but this finally does not result in a productive infection. Previous studies indicate that activation of platelets due to DENV may influence formation of platelet-monocyte aggregates followed by platelet clearance [3]. It is interesting to note that in a nonhuman primate model of DENV infection, DENV-positive platelets were shown to associate with monocytes [5]. We speculate that DENV viremia in the early stages of infection is the primary driver of platelet activation either due to exposure of platelets to DENV (virus attachment or binding) or due to viral entry but not productive infection of platelets. These DENV-exposed/DENV antigen-positive platelets associate with $CD14^+CD16^+$ cells, which may mediate platelet clearance from the circulation. The immune regulation leading to resolution of viremia and associated reduction in $CD14^+CD16^+$ monocytes then help in recovery of circulating platelet numbers. Lower levels of T-cell-platelet complexes observed in this study could be a direct consequence of thrombocytopenia, and this perhaps affects coagulation at the site of injury leading to vascular leakage. A similar mechanism has been proposed in a simian immunodeficiency virus (SIV) animal model, where decreased platelet numbers in SIV infection was shown to be due to platelet-monocyte aggregates, specifically, association of platelets with $CD16^+$ monocytes, and not due to reduced platelet production or destruction mediated by platelet auto-antibodies [17]. Nevertheless, suppression of hematopoiesis has been proposed as a possible cause of transient leukopenia and thrombocytopenia observed in dengue patients and in animal models of dengue infection due to direct infection of bone marrow cells by DENV [18–21].

CONCLUSIONS

Therefore, infection of hematopoietic cells and the ensuing inflammatory response in the bone marrow may act as a trigger to transiently shut down hematopoiesis/thrombopoiesis while the immune system is engaged in clearing the virus from both the bone marrow and in peripheral blood and tissues. Clearance of activated platelets from the circulation by inflammatory/intermediate monocytes may be one of the mechanisms by which homeostasis is maintained and hematopoiesis is resumed. Therefore, our study provides a possible mechanistic insight into the role of viremia, platelet activation, and platelet-monocyte (specifically, $CD14^{+}CD16^{+}$ monocytes) complex formation in thrombocytopenia observed in DENV infections.

Supplementary Data

Supplementary materials are available at *Open Forum Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

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Potential conflicts of interest. All authors: No reported conflicts of interest.

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