Dengue virus binding and replication by platelets

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Key Points

- Platelets replicate and produce infectious DENV.
- DENV binds directly to platelets using DC-SIGN and heparan sulfate proteoglycan as primary receptors.

Dengue virus (DENV) infection causes ~200 million cases of severe flulike illness annually, escalating to life-threatening hemorrhagic fever or shock syndrome in ~500 000. Although thrombocytopenia is typical of both mild and severe diseases, the mechanism triggering platelet reduction is incompletely understood. As a probable initiating event, direct purified DENV-platelet binding was followed in the current study by quantitative reverse transcription-polymerase chain reaction and confirmed antigenically. Approximately 800 viruses specifically bound per platelet at 37°C. Fewer sites were observed at 25°C, the blood bank storage temperature (~350 sites), or 4°C, known to attenuate virus cell entry (~200 sites). Dendritic cell–specific intercellular adhesion molecule–3–grabbing nonintegrin (DC-SIGN) and heparan sulfate proteoglycan were implicated as coreceptors because only the combination of anti–DC-SIGN and low-molecular-weight heparin prevented binding. Interestingly, at 37°C and 25°C, platelets replicated the positive sense single-stranded RNA genome of DENV by up to ~4-fold over 7 days. Further time course experiments demonstrated production of viral NS1 protein, which is known to be highly antigenic in patient serum. The infectivity of DENV intrinsically decayed in vitro, which was moderated by platelet-mediated generation of viable progeny. This was shown using a transcription inhibitor and confirmed by freeze-denatured platelets being incapable of replicating the DENV genome. For the first time, these data demonstrate that platelets directly bind DENV saturably and produce infectious virus. Thus, expression of antigen encoded by DENV is a novel consideration in the pathogen-induced thrombocytopenia mechanism. These results furthermore draw attention to the possibility that platelets may produce permissive RNA viruses in addition to DENV. (Blood. 2015;126(3):378-385)

Introduction

Dengue virus (DENV) is estimated to infect ~390 million people annually, distinguishing it as the most prevalent mosquito-borne pathogen on the globe. Approximately half of infections are asymptomatic, despite potentially high virus titers. The remainder predominantly suffers subclinical, self-limiting flulike symptoms, known as Dengue fever. However, the World Health Organization estimates that ~500 000 cases escalate to life-threatening Dengue hemorrhagic fever or Dengue shock syndrome and require hospitalization, leading to ~20 000 deaths each year. The DENV mosquito vector is endemic to tropical and subtropical climates, placing ~40% of the world’s population at direct risk of infection. However, transfusion transmission, air travel, and global warming firmly establish DENV as an emerging threat to health care systems worldwide, expanding the risk far outside the equatorial zone.

DENV is a Flavivirus and, typical of the genus, has an ~11-kb positive-sense, single-stranded (RNA) genome. The genome encodes a polyprotein that is cleaved posttranslationally into 3 structural (eg, envelope [E] protein) and 7 nonstructural (NS) proteins. The structural proteins form the building blocks of the mature virus, whereas the NS proteins are involved in replication, assembly, and host immune response modulation. Common to all (+)ssRNA viruses, the genome is directly translated in the host cell cytosol by polyribosome complexes. After posttranslational proteolytic maturation of the polyprotein, the virus-encoded RNA-dependent RNA polymerase (NS5) produces negative-sense (−)RNA that acts as a template for feedback-amplified generation of (+)ssRNA that may be subsequently encapsidated for DENV progeny assembly in the Golgi apparatus.

Four DENV serotypes (DENV1-4) share nucleotide sequence homology, but each have unique immunoreactivity. Thus, consecutive infection by different serotypes provides an explanation for progression into clinical pathology, because subneutralizing cross-reactive antibodies may enhance DENV infection via Fc-receptor (FcR)-bearing cells. Understanding how FcRs contribute to DENV host cell attachment and internalization is consequently important in the context of a secondary infection when preexisting heterologous antibodies may enhance DENV infection via FcR-bearing cells. The virus-encoded RNA-dependent RNA polymerase (NS5) produces negative-sense (−)RNA that acts as a template for feedback-amplified generation of (+)ssRNA that may be subsequently encapsidated for DENV progeny assembly in the Golgi apparatus.

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Although essential for hemostasis, the involvement of platelets in inflammation and the host defense against pathogen invasion has been established.13,14 Thrombocytopenia is a hallmark for both fulfille and the more severe forms of DENV disease,15 which in addition to causing bleeding, could be an immune evasion tactic. Vaccinia virus,16 cytomegalovirus,17 HIV,18 influenza virus,19 and hepatitis C virus20 along with DENV are examples of a broad range of virus types that interact with platelets, suggesting a general pathophysiological platelet-virus relationship. The mechanism involved in DENV-mediated thrombocytopenia is multifaceted and incompletely understood, comprising direct and indirect effects on the platelet.15-21 Of note, an early study concluded that binding of platelets to metabolically labeled DENV2 required a virus-specific antibody, but surprisingly could not be inhibited by excess purified Fc.22 This was the paradigm for the platelet-DENV interaction for nearly 2 decades and implied that binding should not exist during an initial infection before virus antibody formation. However, in contrast, a later study employing sensitive atomic force microscopy showed that platelet ultrastructural changes were triggered by DENV2 without the addition of virus-specific antibodies.23 Also, in the absence of added antibodies, DENV2 was recently shown to induce key platelet stimulation markers.24 Although insightful, these studies may be influenced by the effects of cell-culture byproducts in the semipurified virus preparations that were used. The pivotal direct platelet-virus interaction has not yet been ascertained or quantified, which is important to understand in the context of a primary infection where preexisting DENV antibodies do not yet exist.

Platelets are anucleate and consequently lack transcriptional machinery. Nevertheless, they contain cytosolic polyribosomes, rough endoplasmic reticulum, energy-providing mitochondria, and the necessary regulatory factors that enable posttranscriptional and translational mechanisms.25-26 Thus platelets have been shown to synthesize proteins using mature and pre-messenger RNA transferred from mega-karyocytes as a genetic template. Because the platelets of infected patients contain virus-like particles and have been colocalized to virus karyocytes as a genetic template. Because the platelets of infected patients contain virus-like particles and have been colocalized to virus antigen,27 we investigated the hypothesis that platelets are permissive to DENV entry, can translate proteins encoded by the viral (+)ssRNA, facilitate replication of the viral genome, and assemble virus. Furthermore, to address inconsistencies in the literature concerning the requirement for DENV antibodies for association with platelets, we followed direct binding of highly purified DENV to purified platelets. We focused predominantly on DENV2 to be comparable to other published work.22-25 Similar to initial primary DENV infection when no antibody would yet be present, our data for the first time demonstrate saturable binding of DENV to platelets. Suggesting DENV entry and (+)ssRNA decapsidation follows binding to platelets, the virus genome was replicated by several fold at physiological and blood center storage conditions. However, in contrast, a later study employing sensitive methods and techniques showed that platelet-DENV interaction is not dependent on virus antibodies.23 The pivotal direct platelet-virus interaction has not yet been ascertained or quantified, which is important to understand in the context of a primary infection where preexisting DENV antibodies do not yet exist.

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virus. After washing, the virus remaining associated was quantified (10^8 particles/mL) and platelets (10^4 cells/mL) with gentle mixing for 1 hour. To determine the specific bound virus, cells were washed twice in PBS by centrifugation and then incubated for 10 minutes (100 mg/mL) before centrifugation, which is a conventional method to remove specifically bound extracellular virus. After washing, the virus remaining associated was quantified by qRT-PCR and was presumed to be internalized or nonspecifically bound. The specific surface-bound virus was defined as the trypsin-releasable DENV genome copy number, which was calculated by subtracting the DENV genome copy number remaining after trypsin treatment from the total before trypsinization.

DENV-binding to platelets was studied by combining purified virus (10^6 to 10^8 particles/mL) and platelets (10^6 cells/mL) with gentle mixing for 1 hour. To remove unbound virus, cells were washed twice in PBS by centrifugation at 800 g for 5 minutes. Platelet-associated virus was quantified by qRT-PCR. To determine the specific surface-bound DENV, the DENV-platelet mixtures were treated with trypsin for 10 minutes (100 mg/mL) before centrifugation, which is a conventional method to remove specifically bound extracellular virus. After washing, the virus remaining associated was quantified by qRT-PCR and was presumed to be internalized or nonspecifically bound. The specific surface-bound virus was defined as the trypsin-releasable DENV genome copy number, which was calculated by subtracting the DENV genome copy number remaining after trypsin treatment from the total before trypsinization. Binding curves were iteratively fit to a simple rectangular hyperbolic model, which represents a single global class of binding (n = 3, ± standard deviation [SD], smaller than the size of symbols). Inset: Antigenic confirmation of DENV-platelet binding. Purified DENV2 (5 x 10^6 particles/mL) was added to purified platelets (5 x 10^9/mL) and incubated at (A) 4°C, (B) 25°C, or (C) 37°C. Platelets were washed in PBS 3 times. The pellet was solubilized, subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (5% to 20% polyacrylamide), and analyzed for viral E protein antigen. The ~61 kDa monomeric band was quantified by densitometry post- (filled squares) and preinoculation (open squares). The fit of data is arbitrary. A representative western blot is provided.

Western blots
Following polyacrylamide gel electrophoresis of the platelet/DENV proteins, western blot analysis was conducted using anti-DENV E-protein (50 mg/mL) or anti-NS1-protein (50 mg/mL) and horseradish peroxidase–conjugated goat anti-mouse (25 mg/mL) and detected using ECL-Prime chemiluminescent reagent (GE Healthcare). Protein bands were documented using a ChemiGenius 2 analysis system (PerkinElmer) and quantified using GeneTools software. polyvinylidene difluoride was subsequently stained using Coomassie blue G250 to confirm equal loading of the lanes and to standardize immunoblot band intensities.

DENV replication
Platelets (5 x 10^9/mL) were inoculated with purified DENV (5 x 10^6 virus particles/mL) in serum-free M199 for 1 hour at 25°C or 37°C. Freeze-thawed platelets generated by repeating 3 cycles of 80/37°C treatment or mock-inoculated platelets were used as controls. After incubation, the platelet-DENV mixture was spun at 800 g and washed 3 times with PBS to remove unbound virus. The platelet and associated virus was then resuspended in serum-free M199 supplemented with l-glutamine (2 mM), Fungizone, and gentamycin and cultured for 7 days at 25°C or at 37°C in a 5% CO2 atmosphere with gentle continuous agitation. Samples were drawn daily to quantify genome copy number by qRT-PCR for detection of DENV E protein or NS1 antigen by western blot analysis or standard plaque assays on Vero cells.

![Figure 1. Saturable Dengue virus-platelet binding.](https://example.com/figure1.png)

**Figure 1. Saturable Dengue virus-platelet binding.** Purified DENV2 was incubated with a constant number of platelets (5 x 10^9/mL) for 1 hour and then washed 3 times with PBS. Platelet-associated virus was quantified by qRT-PCR. Surface-bound virus (open squares) was determined by subtracting the virus remaining bound after trypsinization from the total virus associated with platelets before trypsin treatment (filled squares). The binding curves were iteratively fit to a simple rectangular hyperbolic model, which represents a single global class of binding (n = 3, ± standard deviation [SD], smaller than the size of symbols). Inset: Antigenic confirmation of DENV-platelet binding. Purified DENV2 (5 x 10^6 particles/mL) was added to purified platelets (5 x 10^9/mL) and incubated at (A) 4°C, (B) 25°C, or (C) 37°C. Platelets were washed in PBS 3 times. The pellet was solubilized, subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (5% to 20% polyacrylamide), and analyzed for viral E protein antigen. The ~61 kDa monomeric band was quantified by densitometry post- (filled squares) and preinoculation (open squares). The fit of data is arbitrary. A representative western blot is provided.

![Figure 2. Dengue virus binding to platelets involves DC-SIGN and HSP.](https://example.com/figure2.png)

**Figure 2. Dengue virus binding to platelets involves DC-SIGN and HSP.** Platelets (5 x 10^9/mL) were incubated for 30 minutes at (A) 4°C, (B) 25°C, or (C) 37°C with PBS, purified Fc (TruStain FcX), nonimmune isotype-matched control IgG, anti-DC-SIGN, LMW heparin, chondroitin sulfate, or a combination of LMWH and anti-DC-SIGN (each at 50 mg/mL). Platelets were then incubated with purified DENV2 (4 x 10^7 particles/mL) for 1 hour. Platelets were washed twice with PBS, and RNA was isolated for quantification of bound virus by qRT-PCR (n = 3, ±SD.).
was appropriate. Binding was also confirmed by this method at 4°C for DENV2 and at 4°C and 25°C for DENV1 (supplemental Figure 2).

**Platelet receptors involved in DENV binding**

Depending on whether direct\(^{35}\) or DENV2-platelet binding was monitored\(^{22}\) or DENV2-induced platelet activation was monitored\(^{23,24}\), there has been inconsistency in the literature concerning the requirement for virus-specific antibodies to facilitate the implied interaction with platelets in vitro. Therefore, the current study strove for preparation of highly purified DENV, which has not been exposed to virus-specific antibodies and is isolated from possible byproducts secreted from the cultured cells used for virus propagation. In agreement with the previously published\(^{35}\) DENV2 direct-binding experiments at 4°C,\(^{22}\) commercially purified Fc (TriStain FcX) had an insignificant effect on the amount of DENV2 bound to platelets (Figure 2). Furthermore, at the higher temperatures used for the reported platelet activation experiments (25°C\(^{22}\) and 37°C\(^{24}\)), the binding of DENV2 was insignificantly affected by Fc. Combined (Figures 1 and 2), the data shown here resolve the inconsistency in the literature and demonstrate conclusively that added virus-specific antibodies are not essential for DENV2 to interact with platelets. The same result was obtained for the other 3 DENV serotypes (supplemental Figure 3).

A monoclonal antibody to DC-SIGN has recently been shown to attenuate the functional modulation of platelets induced by DENV2.\(^{24}\) Here we show that anti-DC-SIGN inhibits primary binding of DENV2 to platelets by ~80% at 4°C, 25°C, and 37°C (Figure 2); more antibody had no further effect (data not shown). In contrast, nonimmune control IgG had an insignificant effect, confirming specificity of the anti-DC-SIGN. Consistent with previous reports for other cell types,\(^{11}\) LMWH also inhibited the DENV-platelet interaction by ~80% (Figure 2), which was specific because the similarly charged polymer...
chondroitin sulfate had no effect. This implicates platelet surface HSP33 as a DENV receptor. Suggesting dual receptor recognition on the platelet surface and combining anti-DC-SIGN and LMWH nearly completely eradicated the sensitive detection of DENV2 binding to platelets by qRT-PCR at each of 3 temperatures. Demonstrating a common platelet-binding mechanism between serotypes, DENV1, 3, and 4 were identically affected by these treatments (supplemental Figure 3).

Because semipurified DENV2 has been reported to cause expression of platelet stimulation markers,24 we measured CD62P expression by flow cytometry under the binding conditions employed here. The baseline signal was corrected using a nonimmune isotype control. After 1 hour at 4°, 25°, or 37°C, 1.8%, 7.9%, and 10.1% of platelets were CD62P positive, respectively. When DENV2 (5 × 10^7 particles/mL) was included during the incubation period, the values increased to 33%, 58%, and 62%. This confirms earlier reports and may suggest that platelet receptors are virus-stimulated to explain the temperature-dependent binding we observed.

**Thrombin-enhanced DENV2 binding to platelets**

To address the possibility that platelet stimulation enhances DENV2, platelets were pretreated with thrombin before the 1 hour incubation with virus at 25°C. Figure 3 shows that the total binding of virus is approximately double at saturation resulting from the effect of thrombin on platelets.

**DENV genome transcription by platelets**

At 25° or 37°C (Figure 1), a significant proportion of DENV2 was resistant to release by excess trypsin and therefore likely internalized. Because platelets have the necessary posttranscriptional and translational machinery to translate the DENV (+)ssRNA genome, which encodes an RNA polymerase, we evaluated DENV genome replication by platelets. After a 1-hour incubation of purified virus with platelets at a ratio of 10:1 to encourage uptake, the platelets were washed and the virus genome copy number was quantified. A 7-day period was evaluated, which encompassed the blood center storage time (5 days) and approached the circulation clearance time (9 to 11 days). Figure 4 shows that the genome of DENV2 was replicated by ~4-fold at 25° and 37°C, which substantiates an earlier preliminary report.34 Mock infected platelets had no detectable DENV RNA as a confirmation of qRT-PCR primer specificity (not shown). The effect of damaging the platelets by a prior freeze-thaw cycle was evaluated. The data demonstrate (Figure 4) that intact platelets are required for enhancement of DENV2 (+)ssRNA copy number. The genome of DENV1, 3, and 4 was comparably replicated by platelets (supplemental Figure 4).

**DENV-encoded protein synthesis by platelets**

Because platelet participation in DENV genome replication was observed, synthesis of a critical DENV protein was next investigated. All Flaviviruses encode the homologous glycoprotein NS1, which is nearly identical for each DENV serotype and is required for viability as part of...
the viral replication complex. We performed western blot analysis using an antibody that recognizes all serotypes. A single 45-kDa antigenic band was detected in the sodium dodecyl sulfate supernatant of platelets infected with DENV2 (Figure 5). This apparent molecular weight is consistent with DENV NS1 that has been proteolytically excised from the precursor polyprotein. The amount of detectable antigen increased over the 7-day experimental period indicating infection-dependent synthesis. Preincubated platelets had no cross-reactive material (open symbol at 0 days). NS1 of DENV1, 3, and 4 was also translated by platelets (supplemental Figure 5).

Production of viable DENV progeny by platelets

Having shown that platelets replicate the DENV2 genome and translate a critical virus-encoded protein, we investigated the possibility that infectious virus is produced. Figure 6 shows that viable DENV2 is highly labile in the presence of platelets and its deterioration is enhanced at 37°C compared with 25°C. The detection of DENV2 plaque-forming units becomes undetectable by 24 hours compared with 72 hours, respectively. To determine if new virus is being produced during this period, the translation elongation inhibitor, cycloheximide, was added to platelets. This resulted in a greatly pronounced decay of infectious DENV, which were immeasurable by 6 hours or 24 hours at 37°C and 25°C, respectively. The difference between experiments without and with cycloheximide represent new virus produced by platelets.

Discussion

DENV infection correlates to thrombocytopenia for both mild and severe forms of the disease. To further understand the multifaceted mechanism that is emerging, we investigated the interaction between highly purified DENV and platelets. In contrast to a previous study, DENV binding to platelets was unambiguously demonstrated in the absence of virus-specific antibodies. Our data are consistent with reports that did not measure binding, but rather platelet marker changes induced by DENV, which also did not require addition of a virus-specific antibody. Several factors may account for the earlier discrepancy, including the virus detection sensitivity, virus strain, or the respective proteolytic maturity of viral M protein. Nevertheless, the data show that DENV may target platelets early in infection before an adaptive immune response. At later stages of infection, when antibodies have been generated, the possibility of antibody-dependent enhancement of binding cannot be excluded.

Similar to other cells types where DC-SIGN and HSP have been separately identified as DENV receptors, we show their involvement on platelets. Interestingly, the inhibitory combination of anti-DC-SIGN and LMWH was best and diminished binding more effectively than either agonist alone (~80%). This suggests dual platelet receptors. Because a subpopulation of platelets express DC-SIGN, the possibility that only a fraction of these coexpress HSP may explain why both anti-DC-SIGN and LMWH were required for complete binding inhibition. It is not yet known whether the 2 extracellular interactions equivalently transmit an outside-in signal or if internalization is required to induce the reported platelet functional state changes, which were partially attenuated by anti-DC-SIGN.

In the current study, ~800 sites per platelet at 37°C were indicated. However, this average stoichiometry may be a low estimate if only the putative subset of permissive platelets is considered. Adding to the initial events leading to DENV-induced thrombocytopenia, thrombin treatment of platelets approximately doubled the number of sites. Therefore, combining treatments to restrict coagulation enzyme activation and receptor engagement by DENV may optimally protect
platelets from resultant thrombocytopenia and progression to severe disease. A model summarizing our findings is shown in Figure 7.

Although platelets do not have a nucleus, they contain the requisite ribosomes, rough endoplasmic reticulum, and mitochondria for synthesis of proteins from an RNA template. Because DENV-like particles have been detected in platelets of infected patients, presumably entering through a mechanism mediated by engagement of DC-SIGN and/or HSP as shown here, replication of the virus (+)ssRNA was evaluated. Indeed, the genome of DENV was amplified several-fold by intact but not freeze-thawed platelets. This observation implicates cell entry, decapsidation, and proteolytic maturation of the translated polyprotein (Figure 7). The viral proteins must be functional for replication, including NS5 and other constituents of the replication complex (NS1, NS2A, NS2B, NS3, NS4A, NS4B) that comprise the viral protease (NS2B/NS3). To clearly demonstrate translation of a DENV protein by platelets, here we show the time-dependent production of NS1 antigen. We selected NS1 because it may be secreted into plasma where it is highly immunogenic and has promising utility as a complementary early DENV infection marker. Furthermore, NS1 relates to thrombocytopenia because anti-NS1 antibodies have been suggested to cross-react with a platelet antigen.

In addition to translational machinery, megakaryocytes transfer Golgi elements to platelets, which is the locale for DENV nucleocapsid assembly and maturation. Therefore, we investigated whether platelets facilitate the generation of infectious DENV progeny. As predicted, the use of a translational inhibitor revealed new virus production by platelets. This greatly prolonged the apparent viability of DENV, which in our in vitro experiment decays over several days depending on temperature. Although it is uncertain if other platelet-binding (+)ssRNA viruses are capable of entry, this gives cause to consider that they may be similarly replicated. Regardless, the data shown here demonstrate that platelets are not merely pathological casualties of infection, but at least for DENV, may also be active contributors to the virus lifecycle.

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Authorship

Contribution: A.Y.S. designed and performed experiments and drafted the manuscript; M.R.S. designed and analyzed experiments and revised the manuscript; and E.L.G.P. designed and analyzed experiments and wrote the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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References


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