PLATELETS AND THROMBOPOIESIS

Dengue virus binding and replication by platelets

Ayo Y. Simon, Michael R. Sutherland, and Edward L. G. Pryzdial

Canadian Blood Services, Centre for Innovation, Ottawa, ON, Canada; and Centre for Blood Research/Department of Pathology and Laboratory Medicine, University of British Columbia, Vancouver, BC, Canada

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 satyrably 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.1,3 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 negative-sense, single-stranded (−ss) RNA genome.4 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 (+ss)RNA viruses, the genome is directly translated in the host cell cytosol by polyribosome complexes.5 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 (+ss)RNA that may be subsequently encapsidated for DENV progeny assembly in the Golgi apparatus.6

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.7,9 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 can facilitate cell interactions. Numerous other putative cell receptors have been identified depending on the cell type, including dendritic cell–specific intercellular adhesion molecule-3–grabbing nonintegrin (DC-SIGN),10 heparan sulfate proteoglycan (HSP),11 and numerous others that recently have been reviewed.12 These may function together on the cell surface to provide avidity effects during primary infection, conceivably depending on the cell type and varying with the activation state of the cell. To further complicate the understanding of DENV-cell interactions, these receptors likely function in concert with FcRs during secondary infection to provide antibody-dependent enhancement of infection.


The online version of this article contains a data supplement.

There is an Inside Blood Commentary on this article in this issue.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked “advertisement” in accordance with 18 USC section 1734.

© 2015 by The American Society of Hematology

378 BLOOD, 16 JULY 2015 • VOLUME 126, NUMBER 3
Although essential for hemostasis, the involvement of platelets in inflammation and the host defense against pathogen invasion has been established. Thrombocytopenia is a hallmark for both fulxile and the more severe forms of DENV disease, which in addition to causing bleeding, could be an immune evasion tactic. Vaccinia virus, cytomegalovirus, HIV, influenza virus, and hepatitis C virus 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. 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. 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. Also, in the absence of added antibodies, DENV2 was recently shown to induce key platelet stimulation markers. 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. Thus platelets have been shown to synthesize proteins using mature and pre-messenger RNA transferred from megakaryocytes as a genetic template. Because the platelets of infected patients contain viruslike particles and have been colocalized to virus antigen, 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. 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 temperatures and duration, but not at 4°C. The production of DENV-encoded NS1 antigen and infectious virus by platelets were also identified. These data provide evidence that platelets contribute directly to the DENV lifecycle and may harbor the pathogen.

**Methods**

**Reagents and proteins**

Tetrasodium ethylenediamine tetracetic acid, chloroform, 2-propanol, low-molecular-weight heparin (LMWH), chondroitin sulfate (Fisher Scientific); N,N,N,N-tetraethylpiperazine-N,N,N,N'-tetraethanesulfonic acid (HEPES), polyethylene glycol 8000 (PEG), bovine serum albumin, cycloheximide (Sigma-Aldrich); and human α-thrombin (Haematologic Technologies) were purchased. Human FcγRI/II/III inhibitor, TruStain FcX (BioLegend), anti-DENV E-protein (MCA2277, AbDseroTec), anti-DENV-NS1 (ab14161, Abcam), CD62P-phycocyanin, immunoglobulin G1 (IgG1)-phycocyanin (Beckman Coulter), anti-hDC-SIGN (mab-hdcsg, InvivoGen), and polyclonal horseradish peroxidase–conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories) were from various sources. Leucocount reagent (BD Bioscience), RNeasy Micro Kit (Qiagen), Turbo DNase (Ambion), iScript advanced cDNA synthesis kit and iTaq universal SYBR green supermix (BIO-RAD), and TRizol reagent and Medium 199 (M199; Life Technologies) were from indicated suppliers.

**DENV purification**

The current study focused on DENV2 (New Guinea C strain). Additional comparison studies among serotypes (DENV1, Hawaii strain; 3, H87 strain and 4, H241 strain) were conducted in some instances and are supplied in the supplemental Materials section on the Blood Web site. All serotypes were kindly provided by Dr Michael Drobet (Viral Zoonoses Laboratory, Public Health Agency of Canada). Confluent African green monkey kidney cells (Vero, CCL-81; ATCC) were inoculated with individual DENV serotypes in serum-free M199 containing bovine serum albumin (1 mg/mL). The infected cell supernatants were collected daily and clarified at 180g for 10 minutes. Virus-containing supernatants were then incubated with PEG (10% v/v) and NaCl (1.5M) overnight at 4°C with gentle mixing. After centrifugation at 8000g for 20 minutes at 4°C, the virus-containing pellets were resuspended in phosphate-buffered saline (PBS), layered onto a stepwise sucrose (10%/50% 60%) gradient and spun at 25 000g for 3.5 hours. The virus band at the 30%/60% sucrose interface was removed, resuspended in PBS, and spun at 25 000g for 1.5 hours. The resulting purified DENV was frozen in PBS at ~80°C in small aliquots. Virus preparations were evaluated for purity by electron microscopy and quantified using a latex sphere standard as previously described. To derive the virus particle number per milliliter using an alternate method that was determined comparable to electron microscopy, total viral RNA was extracted for quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis using the reported molecular weight of the genome RNA (3.3 × 10^6). The DENV viability was measured by Vero cell infectivity using standard plaque assays. Newly thawed virus aliquots were used for each experiment.

**Platelet preparation**

Washed human platelets from healthy donors were obtained under approved informed consent. Blood was drawn into Vacutainers containing citrate (BD Bioscience), centrifuged at 300g for 20 minutes at room temperature to obtain platelet rich plasma, and then further centrifuged at 800g for 10 minutes at room temperature. The pellet was gently resuspended and washed 3 times in 20 mM HEPES, 150 mM NaCl, 0.1% PEG (HEPES-buffered saline/PEG, pH 7.4) in plastic tubes at room temperature. Platelet concentration, the extent of activation (CD62P), and the contaminating leukocyte concentration (CD41 antigen; Leucocount) were measured by flow cytometry. Less than 10% of platelets expressed CD62P antigen, whereas leukocytes comprised fewer than 1 per reaction mixture (ie, <1 leukocyte: 10 000 platelets).

**Dengue virus RNA quantification**

Two-step qRT-PCR was conducted as previously described except DENV RNA was extracted from homogenized platelet pellets using TRizol reagent. DENV RNA was purified with RNeasy Micro Kit and further incubated with Turbo DNase to digest and eliminate DNA. DENV RNA samples (1 μg) were converted into first-strand complementary DNAs at 42°C for 30 minutes, then 85°C for 5 minutes using the iScript advanced cDNA synthesis kit. qRT-PCR was performed using iTaq universal SYBR green supermix with denaturation at 95°C for 30 seconds, followed by 40 cycles of amplification at 95°C for 15 seconds, and melting curve analysis at 60°C at 1 minute conducted on an Applied Biosystems model 7900HT. The number of RNA genome copies was determined using standard plaque assays. Newly thawed virus aliquots were used for each experiment.

**VOLUME 126, NUMBER 3 PLATELETS REPLICATE DENGUE VIRUS 379**

**Blood, 16 July 2015 • Volume 126, Number 3 Platelets Replicate Dengue Virus**

From www.bloodjournal.org by guest on April 20, 2017. For personal use only.
After washing, the virus remaining associated 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 × 10^6 particles/mL) was added to purified platelets (5 × 10^5/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 1. Saturable Dengue virus-platelet binding. Purified DENV2 was incubated with a constant number of platelets (5 × 10^5/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 DENV2-platelet binding. Purified DENV2 (5 × 10^6 particles/mL) was added to purified platelets (5 × 10^5/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.

DENV-platelet binding

DENV binding to platelets was studied by combining purified virus (10^6 to 10^8 particles/mL) and platelets (10^5 cells/mL) with gentle mixing for 1 hour. To remove unbound virus, cells were washed twice in PBS by centrifugation at 800g 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 μg/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 trypsin. Binding curves were iteratively fit to a rectangular hyperbola as the simplest model representing a single class of binding sites to estimate virus-platelet stoichiometry using GraphPad.

Western blots

Following polyacrylamide gel electrophoresis of the platelet/DENV proteins, western blot analysis was conducted using anti-DENV E-protein (50 ng/mL) or anti-NS1-protein (50 ng/mL) and horseradish peroxidase–conjugated goat anti-mouse (25 ng/mL) and detected using ECL-Prime chemiluminescent reagent (GE Healthcare). Protein bands were documented using a ChemiGenius2 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 × 10^5/mL) were inoculated with purified DENV (5 × 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 800g 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 gentamicin 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.
Results

DENV binds to platelets without viral antibody

To investigate the direct DENV2-platelet interaction, virus purified by sucrose density ultracentrifugation and platelets, containing an insignificant number of leukocytes measured by flow cytometry, were used. The number of DENV2 genome copies was quantified by qRT-PCR to follow virus particles bound. This method was validated by electron microscopy in which standardized latex spheres were added as an internal control (not shown). Purified DENV2 associated with the platelet (Figure 1). At 4°C, cell membranes are insufficiently fluid to facilitate endocytic or fusogenic processes over the experimental timeframe; therefore, treatment with trypsin completely removed platelet-associated virus. This further demonstrated that insignificant nonspecific binding occurs to platelets, which would not be removed by trypsin. The DENV2 that was bound to the platelet was iteratively fit to a simple rectangular hyperbolic model ($r^2 > 0.97$), which represents a single global class of binding. The predicted number of sites per platelet at saturation for DENV2 at 4°C was ~200 regardless of trypsin treatment.

When blood is donated for transfusion, platelet-rich units are subsequently stored at 25°C. Figure 1 shows that DENV2 binds to platelets at this temperature, although with somewhat less efficacy than at the physiological temperature (37°C). Unlike the 4°C interaction, at these higher temperatures trypsinization did not remove all of the platelet-associated DENV2, indicating internalized virus. The predicted number of trypsin-corrected surface binding sites for DENV2 at saturation was ~300 sites/platelet at 25°C and ~800 sites/platelet at 37°C. To achieve apparent half-maximal saturation required considerably lower concentrations of virus as temperature was increased. The obvious trend toward more binding sites at the higher temperatures may indicate contributions of activation-dependent binding site expression in addition to the anticipated thermodynamic factors. DENV bound with very similar parameters across serotypes (supplemental Figure 1).

Analogous to an earlier published protocol, we followed DENV structural E protein antigen by western blot as an independent probe for platelet binding at 25°C over time. This method confirmed the binding shown by the qRT-PCR procedure. Densitometric estimation of the respective ~61-kDa band intensities (Figure 1, inset) further illustrated that the 60-minute incubation period used in the following experiment 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 $^{35}$S]DENV2-platelet binding or DENV2-induced platelet activation was monitored, there has been inconsistency in the literature concerning the requirement for added 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 $^{35}$S]DENV2 direct-binding experiments at 4°C, 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$^{23}$ 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. Here we show that anti-DC-SIGN inhibits primary binding of DENV2 to platelets by ~80% at 4°, 25°, 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, 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, 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°C, 25°C, 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°C 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°C and 37°C, which substantiates an earlier preliminary report. 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. Preinoculated 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 (~100%) 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 req-
quisite ribosomes, rough endoplasmic reticulum, and mitochondria
for synthesis of proteins from an RNA template.26 Because DENV-
like particles have been detected in platelets of infected patients,27
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
implies 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 immunogenic38 and
has promising utility as a complementary early DENV infection
marker.39,40 Furthermore, NS1 relates to thrombocytopenia because
anti-NS1 antibodies have been suggested to cross-react with a
platelet antigen.41,42 The current study provides evidence that
platelets can independently synthesize NS1. Because NS1 may be
retained by the infected cell membrane, it is conceivable that direct
viral antigen production may impact platelet clearance by immune
complex formation. Blocking the interaction between antibodies and
putative platelet-bound NS1 may be a further strategy to mitigate
thrombocytopenia.

In addition to translational machinery, megakaryocytes transfer
Golgi elements to platelets,43 which is the locale for DENV nucle-
}

References

496(4744):504-507.
spread of dengue virus types: mapping the
70 year history. Trends Microbiol. 2014;22(3):
138-146.
4. Noble CG, Shi PY. Structural biology of dengue
virus enzymes: towards rational design of
5. Rodenhuis-Zybert IA, Wilschut J, Smit JM.
Dengue virus life cycle: viral and host factors
67(16):2773-2786.
6. Urcuqui-Inchima S, Patino C, Torres S, Haenni
A-L, Diaz FJ. Recent developments in understanding
7. Littaua R, Kurane I, Ennis FA. Human IgG Fc
receptor II mediates antibody-dependent
enhancement of dengue virus infection. J Immunol.
1990;144(3):3183-3186.
8. Dejjaittaiwi W, Jummainsong A, Onsirisakul N,
et al. Cross-reacting antibodies enhance dengue
virus infection in humans. Science. 2010;
328(5979):745-748.
9. Guzman MG, Alvarez M, Halstead SB. Secondary
infection as a risk factor for dengue hemorrhagic
fever/dengue shock syndrome: an historical
perspective and role of antibody-dependent
158(10):1445-1459.
10. Tassaneentheep B, Burgess TH, Granelli-Piperno
A, et al. DC-SIGN (CD209) mediates dengue
virus infection of human dendritic cells. J Exp
virus infectivity depends on envelope protein
binding to target cell heparan sulfate. Nat Med.
1997;3(8):866-871.
12. Alen MM, Schols D. Dengue virus entry as target
629475.
13. Projan D, Koenen RR. Platelets: key players in
1167-1175.
2011;11(4):264-274.
15. Holtz E, Toller ND, Zimmerman GA, Weyrich AS,
Bozza FA. Platelets in dengue infection.
16. Bzik T, Sarov I, Livne A. Interaction between
1982;59(3):482-487.
17. Agbayo FR, Wasi S. Human cytomegalovirus
interaction with platelets and adhesive
glycoproteins: significance in viral pathogenesis.
Thrombocytopenia and AIDS: possible direct role
19. Terada H, Baldini M, Ebbe S, Madoff MA.
Interaction of influenza virus with blood platelets.
20. Haama S, Li C, Allain JP. The dynamics of
hepatitis C virus binding to platelets and 2
mononuclear cell lines. Blood. 2001;98(8):
2293-2300.
contributing to the disturbance of coagulation and
fibrinolysis in dengue virus infection. J Formos
22. Wang S, He R, Patarapolikul J, Innis BL,
Anderson R. Antibody-enhanced binding of
interaction between dengue 2 virus and human
blood platelets using atomic force and electron
57(3):113-118.
induces platelet activation, mitochondrial
dysfunction and cell death through mechanisms
that involve DC-SIGN and caspases. J Thromb
fibrinolysis in dengue virus infection.
26. Hama S, Li C, Allain JP. The dynamics of
hepatitis C virus binding to platelets and 2
mononuclear cell lines. Blood. 2001;98(8):
2293-2300.
contributing to the disturbance of coagulation and
fibrinolysis in dengue virus infection. J Formos
28. Wang S, He R, Patarapolikul J, Innis BL,
Anderson R. Antibody-enhanced binding of
interaction between dengue 2 virus and human
blood platelets using atomic force and electron
57(3):113-118.
induces platelet activation, mitochondrial
dysfunction and cell death through mechanisms
that involve DC-SIGN and caspases. J Thromb
fibrinolysis in dengue virus infection.


Dengue virus binding and replication by platelets

Ayo Y. Simon, Michael R. Sutherland and Edward L. G. Pryzdial