Platelets in circulation normally do not adhere to resting endothelial cells. However, in response to vascular injury they adhere to stimulated endothelium and thereby play an essential role in hemostasis and thrombosis. Infection with dengue-2 virus can cause illness accompanied by thrombocytopenia and hemorrhage. Increased adherence of platelets to stimulated endothelial cells could contribute to the thrombocytopenia. In this study, adherence of radioisotopically labeled platelets to 1) unstimulated, 2) lipopolysaccharide (LPS)-stimulated, and 3) dengue-2 virus-infected human umbilical vein endothelial cells (HUVEC) were measured in an in vitro assay. Primary HUVEC were cultured in 96-well tissue culture plates in the presence or absence of LPS or dengue-2 virus. These cells were co-incubated with 3H-adenine-labeled fresh platelets for 30 min after which the cells were assayed for adherent platelets. Within 30 min there was maximum adherence of platelets to confluent LPS-stimulated HUVEC (36 ± 4% over controls; P = 0.005). In comparison, there was a significant increase in adherence to dengue-2 infected HUVEC (78 ± 7%; P ≤ 0.001). Additionally, platelet adherence was visualized using fluorescent microscopy. Dengue-2 infection stimulated the HUVEC as monitored by expression of E-selectin. Platelets that adhered to dengue-2 or LPS-stimulated HUVEC were activated as visualized by dual fluorescent probes. These data demonstrate that human platelets adhere to dengue-2 virus-stimulated HUVEC and this interaction could contribute to the thrombocytopenia observed during infection.

INTRODUCTION

Vascular endothelial cells are important regulators of thrombosis and hemostasis. They actively influence not only the plasma coagulation system but platelet function as well. Under normal flow conditions, the endothelial cells do not act as a thrombogenic surface; thus, platelets do not adhere to them. However, under certain conditions they may become procoagulant and cause increased adherence of platelets, promoting increased thrombosis.1 For instance, when stimulated with endotoxin, interleukin-1, or tumor necrosis factor, endothelial cells synthesize tissue factor and other procoagulant proteins, which may cause thrombosis in patients with chronic inflammatory diseases.

A wide variety of adhesion molecules are expressed on stimulated endothelial cells, including two selectins, P-selectin and E-selectin, that are involved in interactions with leukocytes and platelets. P-selectin is stored in the Weibel-Palade bodies in the endothelial cells as well as in the α-granules of the platelets. Expression of E-selectin is induced when the cells are stimulated with cytokines.2 During platelet-endothelial interaction in inflamed mesenteric venules, both P-selectin and E-selectins are upregulated.3 Several investigators have reported platelet adhesion to thrombin-treated endothelial cells.4–7 Previous studies have shown the adherence of platelets to injured,8,9 or virally transformed10 endothelial cells.

Decreased platelet function and number may contribute to the severity of disease in some viral infections. One such disease is dengue, which is caused by a flavivirus transmitted by the Aedes aegyptimosquito. Infection with this virus leads to illness with various degrees of severity. One predominant feature of dengue is thrombocytopenia. Dengue virus appears to suppress marrow production of platelets: this may contribute to bleeding early in infection.11,12 However, just as platelet counts reach their nadir, marrow production of platelets resumes. Interaction of platelets with the endothelium could contribute to this thrombocytopenia. Most thrombocytopenia appears to result from peripheral use and platelet destruction. We have previously shown that the coagulopathic abnormalities are well compensated and hemorrhage in dengue is most likely due to activation of platelets.3,4 Therefore, vascular alteration may be the principal factor involved in the association of thrombocytopenia and hemorrhage with disease severity.

We have used a sensitive assay to measure adhesion of radioisotopically labeled platelets to cultured human umbilical vein endothelial cells (HUVEC).10 Our results show that stimulation of endothelial cells by dengue-2 virus leads to increased adherence of platelets. This phenomenon could explain the thrombocytopenia observed in the disease.

MATERIALS AND METHODS

Endothelial cells. Primary human endothelial cells isolated from umbilical cord vein were purchased from Clonetics (Walkerville, MD). Cells were cultured using endothelial growth media (EGM2) media and grown in 75 cm2 culture flasks (Corning, Cambridge, MA) until 70–90% confluence in a 37°C, 5% CO2 humidified incubator. Confluent, subcultured cells in 96-well plates were stimulated using 3 or 10 μg/ml of lipopolysaccharide (LPS) (Sigma, St Louis, MO) for 4 hr at 37°C in the 5% CO2 humidified incubator and washed once with Hanks’ balanced salt solution (HBSS) containing Mg2+ and Ca2+ (Sigma) prior to the adhesion assay.

Dengue virus (Dengue type 2 S16803)13-infected cells were incubated with a multiplicity of infection (MOI) ranging from 0.1 to 10 for 1 hr at 37°C in a 5% CO2 incubator. The cells were then replenished with new media and incubated for 24, 48, 72, or 96 hr. The cells were washed once with HBSS containing Mg2+ and Ca2+ prior to the adhesion assay. Supernatants were collected from the plates infected for 48 hr with dengue-2 virus (MOI = 1) for detection of viral proliferation. Plaque-forming units were 1.5 ± 1.1 × 105. The media were changed at 48 hr for the plates infected for 72 hr and 96 hr.

Isolation and labeling of platelets. This was performed according to the method described by Curwen and others.10 Blood was collected from a healthy donor in 3.8% sodium citrate. The blood was centrifuged for 10 min at 1,300 revolutions per minute (rpm) (338 × g) and the platelet-rich plasma (PRP) was collected. The PRP was labeled with 15 μCi/ml of 3H-adenine (New England Nuclear Research Prod-
uants, Reston, VA) for 45 min at 22°C. Half of the labeled PRP was centrifuged at 3,000 rpm (1,875 × g) for 10 min to obtain the platelet-poor plasma (PPP). To prevent the uptake of unincorporated plasma 3H-adenine from PRP by endothelial cells during adhesion assays, a 100-fold excess of nonradioactive adenine (0.1 mM final concentration) was added to labeled PRP and PPP 10 min prior to incubation with the cells.

**Adhesion assay.** Confluent cells in the 96-well plate were washed once with HBSS containing Mg2+ and Ca2+ at 37°C. Labeled PRP (0.2 ml) or PPP were added to wells and incubated at 37°C in a 5% CO2 humidified incubator for 30 min. Wells were washed three times with HBSS without Ca2+ and Mg2+ (Sigma). Cells were removed by the addition of 0.2 ml of trypsin (0.05%) and EDTA (0.02%) in Pucks Saline A (Sigma) for 30 min at 37°C. The endothelial cells and adherent platelets were collected along with the three washes into vials containing 10 ml of an aqueous scintillation fluid (Pico-Fluor 15; Packard, Meriden, CT) and counted in a beta scintillation counter (2500 TR; Packard Instrument Co., Downers Grove, IL).

**Fluorescence microscopy.** Endothelial cells grown to confluence on 22 mm2 glass cover slips were incubated with fresh platelets for 30 min at 37°C in a 5% CO2 incubator. The cover slips were washed in phosphate-buffered saline to remove the non-adhered platelets.

Cells were labeled with fluorescein isothiocyanate (FITC)-labeled anti-CD42a antibody (marker for platelets), phycoerythrin-labeled anti-CD62a antibody (marker for activated platelets and endothelial cells), or biotin-labeled E-selectin (marker for stimulated endothelial cells) for 30 min at 22°C, washed in phosphate-buffered saline, and visualized with a Texas red streptavidin reagent (Pierce, Rockford, IL). To detect dengue-2 virus-infected cells, the HUVEC were labeled with anti-dengue-2 antibody (a mouse monoclonal antibody produced by the Department of Virology of Walter Reed Army Institute of Research), followed by an FITC-labeled secondary goat anti-mouse antibody. The cells were fixed with 2% paraformaldehyde for 15 min 22°C.

A Leitz Orthoplan (Leica, Deerfield, IL) microscope with a 40× objective was used. Images were collected with a color digital camera (DEI-470; Optronics Engineering, Goleta, CA) and a computer (Macintosh; Apple Computers, Cupertino, CA).

**Calculations and statistical analysis.** The percentage incorporation of 3H-adenine by platelets was calculated using the formula (cpm/μl of PRP)-(cpm/μl of PPP)/(cpm/μl of PRP). Incorporation of 3H-adenine ranged from 70% to 90%. The labeled PPP was used to determine the amount of 3H-adenine taken up by the endothelial cells in the absence of platelet adhesion. Adherence was determined using stimulated 3H-adenine uptake by endothelial cells with adherent platelet (PRP) minus endothelial cells in the absence of platelets (PPP). Data are expressed as percentage increase using stimulated endothelial cells over non-stimulated cells (control). Values are reported as the mean ± SEM and significance was determined by a two-tailed Student’s t-test.

**RESULTS**

The HUVEC treated with either LPS (3 μg/ml) for 4 hr or dengue-2 virus (MOI = 1) for 48 hr were incubated with freshly obtained healthy human donor platelets for 10, 30, 60, and 120 min to determine the percentage adherence over non-stimulated, control endothelial cells. Figure 1 shows that maximum adherence occurred at 30 min of co-incubation. Platelet adherence to dengue-2 virus-treated endothelial cells (78 ± 7%; P = 0.05) was significantly higher (2.1 fold; P = 0.001) than LPS-treated endothelial cells (36 ± 4%) at 30 min. Platelets appeared to detach from the endothelial cells when incubated for longer than 30 min especially when treated with the virus.

When one-day-old apheresed single donor platelets were incubated with LPS-treated endothelial cells, platelet adherence was reduced by 47% (17 ± 4%) compared with freshly obtained platelets (36 ± 4%; P = 0.07). Thus, freshly obtained platelets were used regularly in subsequent experiments.

Platelet adherence (%) was compared in cells treated with two different concentrations of LPS (3 and 10 μg/ml), tumor necrosis factor-α (TNF-α, 2 μg/ml), and dengue-2 virus at an MOI of 1. Endothelial cells were incubated with the virus for 48 hr and with LPS or TNF-α for 4 hr prior to incubation with the platelets for 30 min. Figure 2 shows that the % adherence was maximum with the dengue-2 virus (78 ± 7%; P = 0.001 over LPS at 3 μg/ml), followed by LPS at a concentration of 3 μg/ml (36 ± 4%). Adherence was reduced at the higher concentration of LPS (22.8 ± 0.8%) and with TNF-α (17.6 ± 12%).

**Infection of HUVEC with dengue-2 virus.** The HUVEC were infected with dengue-2 virus for 24, 48, 72, and 96 hr at 37°C. After gentle washing, they were co-incubated with platelets for 30 min. Figure 3A shows that the cells incubated with the virus for 48 hr showed maximum adherence (78 ± 7%). Platelet adherence was variable at 72 hr post-infection.

![Figure 1. Time kinetics of adherence of human platelets to human umbilical vein endothelial cells (HUVEC). Untreated or HUVEC treated with either lipopolysaccharide (LPS) (3 μg/ml, n = 25) for 4 hr or dengue-2 virus (multiplicity of infection = 1, n = 12) for 48 hr were incubated with 3H-adenine-labeled platelets freshly obtained from a healthy donor. At indicated times, nonadhered platelets were washed off and the percentage of platelets adhering to the treated HUVEC was determined using the non-stimulated HUVEC as controls. Bars show the mean ± SEM.](image)
By 96 hr post-infection, the HUVEC were incapable of sustaining a detectable platelet interaction.

Increasing the MOI of the virus from 0.1 to 1.0 resulted in a significant increase \((P < 0.001)\) in the percentage of platelets that adhered \((\text{MOI} = 0.1; 12.3 \pm 0.9\% \text{ to } \text{MOI} = 1; 78 \pm 7\%)\) (Figure 3B). However, increasing the MOI from 1.0 to 10.0 \((81 \pm 0.6\%)\) did not increase the adherence of platelets \((P = 0.62)\).

**Evidence for platelet adherence using fluorescent markers.** Human platelets can be visualized on endothelial cells if one uses antibodies against FITC-labeled CD42a that recognize glycoprotein IX (GPIX). There are practically no labeled platelets seen in Figure 4A and B when incubated with untreated endothelial cells. In contrast, labeled platelets adhered to endothelial cells pre-treated with LPS (Figure 4D), as confirmed by the fluorescent image (Figure 4C).

To determine the activation state of platelets adhering to dengue-2 virus-treated HUVEC, the platelets were double-labeled with FITC-labeled anti CD42a (green) and phycoerythrin-labeled anti CD62a (red). Adherence of platelets was observed in the light image (Figure 5C) and this was confirmed by the anti-CD42a label in Figure 5A. The platelets that adhered were activated as seen in Figure 5B. Furthermore, as expected, the antibody CD62a labeled the endothelial cells as well, indicating that both the endothelial cells and the platelets were activated in the process of adhesion.

The HUVEC were labeled with E-selectin (a marker of endothelial activation) to determine if dengue-2 virus stimulated the cells (Figure 6). When the cells were pre-treated with either dengue-2 virus \((\text{MOI} = 1)\), LPS \((3 \mu\text{g/ml})\), or TNF-\(\alpha\) \((2 \mu\text{g/ml})\), nearly all the cells were labeled, clearly indicating that they were activated. In contrast, the control cells were not stimulated (Figure 6A and B).

To confirm infection of the HUVEC with the dengue-2 virus, the cells were labeled with FITC-labeled anti-dengue-2 IgG. The non-infected cells were totally unlabeled (Figure 7C and D). Dengue-2 virus-infected HUVEC were brightly labeled with the antibody, indicating infection (Figure 7A and B). However, upon increasing the MOI from 1 to 10, there was no apparent increase in the infection (Figure 7E and F).

**DISCUSSION**

Platelets do not adhere to endothelial covering; however, when the endothelial lining of a blood vessel is damaged due to vascular injury or altered by viral transformation, platelets...
do adhere to the exposed subendothelial surface. We have previously shown that patients infected with dengue virus who develop dengue fever or dengue hemorrhagic fever have marked thrombocytopenia. In an effort to explain the causes of thrombocytopenia in dengue-infected patients, we developed an in vitro model to show an increased adherence of platelets to dengue virus-infected endothelial cells.

The clinical manifestations of dengue are fever and hemorrhagic diathesis. The hemorrhagic manifestations are related to several factors including thrombocytopenia. The mechanism of thrombocytopenia is still not well understood. Many elegant investigations have been conducted to describe the coagulopathy, platelet functional defects, and shortened survival, and vasculopathy that occur to various degrees during severe cases of dengue. Destruction of platelets appears to occur because of complement activation (presumably because platelets bind virus antigens) and also because of peripheral use. In a recent study involving Thai children hospitalized with dengue fever and dengue hemorrhagic fever, we hypothesized that during dengue infection, a major fraction of circulating platelets have been activated. As a consequence, the platelets are either removed from circulation or lose the ability to promote clot formation. Our data suggest that vascular alteration may be the principal factor involved in the association of thrombocytopenia and hemorrhage with disease severity.

Several reports in the literature have investigated why platelets normally do not adhere to the endothelial lining of the blood vessels. Platelet inactivation by endothelial prostaglandin PGI₂ has been assumed to be responsible for the lack of platelet adherence. However, this hypothesis was not supported by the work of some investigators such as Curwen and others, and the role of PGI₂ in preventing platelet ad-
hesion remains unclear. Factors such as fibronectin and heparin-like glycosaminoglycans need to be further evaluated.

Nonetheless, many studies have demonstrated that platelets do adhere to cultured vascular endothelial cells in the presence of thrombin. Observations with video-enhanced contrast microscopy have suggested that thrombin may be involved in the endothelial damage and formation of platelet thrombi on the endothelial cells after disturbance of blood flow. Moreover, an increase in F, the prothrombin fragment, is indicative of an increase in generation of thrombin, which could also lead to an activation of endothelial cells and platelets.

It has been reported that the dengue virus titers increased rapidly until 48 hr after an application of a culture medium containing dengue virus to the cells. Maximum adherence of platelets to the endothelial cells also occurred in our system after 48 hr of infection. In concurrence with our work, Curwen and others showed maximum adherence of platelets to SV40-transformed HUVEC at 30 min, after which adherence decreased. Furthermore, dengue-2 virus infection of HUVEC in our laboratory significantly increased the adherence of platelets in comparison with LPS and TNF-α. In a study by Funahara and others, dengue virus-infected endothelial cells incubated with platelets showed a decrease in residual platelet count within 10 min. It is conceivable that different ligands and/or triggers are stimulated under different inflammatory conditions. Stimulated expression of interleukin-1, TNF-α, and platelet-activating factor may play a role in regulating adherence of cells from the bloodstream.

With the use of fluorescent markers such as CD42a and CD62a, we were able to show that the platelets adhering to the endothelial cells were activated. During inflammation, adhesion molecules such as E-selectin, P-selectin, vascular cell adhesion molecule-1, and intercellular adhesion molecules are induced and/or upregulated on endothelial cells. In our system, the endothelial cells were also stimulated since they were concurrently labeled with antibodies specific for P- and E-selectins. Ligands for E- and P-selectins are constitutively expressed on the surface of platelets. Thus, it is possible that platelet and endothelial selectins together reinforce sufficient interactions to allow platelets to participate in inflammatory processes.

FIGURE 6. Determination of the activation state of human umbilical vein endothelial cells (HUVEC) after infection with dengue-2 virus. Unstimulated (A and B) or HUVEC stimulated with dengue-2 virus (multiplicity of infection = 1; C and D), lipopolysaccharide (3 μg/ml) (E and F) or tumor necrosis factor-α (2 μg/ml) (G and H) were labeled with E-selectin, a marker of endothelial activation. The fluorescent images can be visualized in A, C, E, and G and the corresponding light images in B, D, F, and H. Bars represent 10 μm.

FIGURE 7. Dengue-2 virus infection visualized by antibody to dengue-2 virus. Human umbilical vein endothelial cells (HUVEC) infected with dengue-2 virus were labeled with fluorescein isothiocyanate-labeled anti-dengue-2 IgG. Untreated cells (C and D; magnification ×16), virus-infected cells (multiplicity of infection [MOI] = 1, A and B; magnification ×40). Cells can be visualized with their fluorescent labels in A and C, with the corresponding light images in B and D. The scale bar for A, B, C, and D represents 10 μm. HUVEC infected with dengue-2 virus are shown at a lower magnification of ×4 at an MOI of 1 (E) and 10 (F). Bars represent 40 μm.
We have shown by using fluorescent E-selectin that nearly all HUVEC were activated. However, only isolated islands of cells were labeled with fluorescent anti-dengue-2 IgG at an MOI of either 1 or 10 (Figure 7). Thus, it is possible that once the HUVEC are stimulated by a requisite MOI of dengue-2 virus (such as an MOI of 1), they are sufficiently stimulated to cause the release of agents, such as cytokines, that would subsequently activate the platelets and increase their adherence to the endothelium. Thus, while an MOI of 0.1 might not be adequate, an MOI of 1 may be just as efficient as an MOI of 10 to sufficiently stimulate the HUVEC. Our in vitro experiments showed an increase in adherence from an MOI of 0.1 to 1.0. However, adherence was unaltered when the MOI increased from 1 to 10, indicating that an MOI of 1 results in a level of infection such that the infected cells can then secrete a sufficient level of cytokines that can stimulate the whole culture.

In our present study, we have demonstrated dengue-2 virus infection of endothelial cells by visualizing dengue-2 viral antigen in endothelial cells using anti-dengue-2 virus antibodies. Our data show that stimulation of endothelial cells by dengue-2 virus increased adherence of platelets. This phenomenon could explain the thrombocytopenia observed in the disease. Future studies involving other strains of the dengue virus would be important in demonstrating that this phenomenon is not limited to this one strain. Furthermore, inactivated virus would be important in demonstrating that this phenomenon could explain the thrombocytopenia observed in the disease.


Acknowledgments: We are extremely grateful for the generous supply of virus (dengue-2) and the dengue-2 antibody provided by Dr. Niranjan Kanesa-Thasan and Jeanne M. Burrous (Department of Virus Diseases, Walter Reed Army Institute of Research, Silver Spring, MD).

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