Platelet Activation and Platelet-Monocyte Aggregate Formation Contribute to Decreased Platelet Count During Acute Simian Immunodeficiency Virus Infection in Pig-tailed Macaques

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Platelets are key participants in innate immune responses to pathogens. As a decrease in circulating platelet count is one of the initial hematologic indicators of human immunodeficiency virus (HIV) infection, we sought to determine whether decline in platelet number during acute infection results from decreased production, increased antibody-mediated destruction, or increased platelet activation in a simian immunodeficiency virus (SIV)/macaque model. During acute SIV infection, circulating platelets were activated with increased surface expression of P-selectin, CD40L and major histocompatibility complex class I. Platelet production was maintained and platelet autoantibodies were not detected during acute infection. Concurrent with a decrease in platelet numbers and an increase in circulating monocytes, platelets were found sequestered in platelet-monocyte aggregates, thereby contributing to the decline in platelet counts. Because the majority of circulating CD16+ monocytes formed complexes with platelets during acute SIV infection, a decreased platelet count may represent platelet participation in the innate immune response to HIV.

Keywords: platelet activation; thrombocytopenia; HIV; SIV; macaque; platelet-monocyte aggregate; CD16+ monocyte.

Platelet decline was recognized as a clinical sign of AIDS before the identification of human immunodeficiency virus (HIV) as the causal lentivirus [1]. Low platelet counts have since been associated with HIV disease progression, including high plasma viral load [2], low CD4+ T-cell counts [3], immune reconstitution inflammatory syndrome [4], and central nervous system damage [5, 6]. However, despite this substantial body of evidence that a decrease in platelet numbers either contributes to or results from HIV infection, the cause of decreased platelet count has yet to be identified.

In general, a decline in platelet count is caused by decreased production of platelets, increased antibody-mediated destruction of platelets, or increased activation and subsequent sequestration of platelets in cell–cell interactions [7]. Previous studies of HIV-infected individuals provided evidence for decreased, increased, and no change in platelet production [8, 9]. Reports of fewer megakaryocytes in bone marrow of infected individuals are countered by reports of sufficient numbers [10, 11]. Similarly, platelet autoantibodies are detected in some but not all HIV-infected individuals [8]. These antibodies...
target glycoproteins on the HIV envelope that share homology with platelet surface markers [12] and thereby increase the rate of platelet destruction and decline during HIV infection [13].

An alternative explanation for the decline in platelet count during HIV infection is that activated platelets subsequently bind to leukocytes, such as monocytes and lymphocytes, or to endothelial cells and thus are removed from circulation. Platelets participate in the immune response by interacting with these cells through cell-cell interactions and cytokine signaling [14]. Platelet activation, represented by increased expression of platelet surface markers, such as P-selectin [15–17], or by increased serum levels of soluble cytokines released upon platelet activation [18–20], has been reported in lentivirus infection. Platelet-monocyte aggregates (also known as platelet-monocyte complexes or monocyte-platelet complexes/aggregates) have been reported in asymptomatic HIV-infected individuals [16] and provide evidence for both platelet activation and platelet participation in the immune response to HIV.

Previous work to define causes of HIV-associated thrombocytopenia has been complicated by variable or undefined clinical stage of infection, virus strain, and treatment history. HIV-infected individuals progress through acute, asymptomatic, and terminal stages of infection. The acute phase, which spans the first 100 days after infection, is characterized by an increased plasma viral load, decreasing CD4+ T-cell counts, and a marked proinflammatory cytokine response, with the severity of these events peaking within the first month [21]. Acute infection is difficult to study in humans because patients typically present after this clinical stage. The simian immunodeficiency virus (SIV)/macaque model of HIV infection also demonstrates acute, asymptomatic, and terminal stages of infection [22] and is valuable for studying the earliest events of SIV infection while controlling for virus strain and treatment [4].

We sought to define the cause of the decline in platelet count during acute infection in a well-characterized SIV/macaque model of HIV infection [22]. We have found that the decline in platelet count is biphasic, with decreases during both acute and asymptomatic infection. During acute SIV infection, increased platelet activation occurred concurrently with a marked increase in platelet binding to monocytes, with the highest percentage of platelet-monocyte aggregates found in CD16+ monocyte subsets. Because platelet production was maintained and platelet autoantibodies were not detectable, platelet activation and subsequent interactions with circulating monocytes contributed to the decreased platelet count during acute SIV infection.

METHODS

Animals
Juvenile male pig-tailed macaques (Macaca nemestrina) were inoculated intravenously with SIV/17E-Fr and SIV/DeltaB670 or with saline (uninfected controls) [22]. All macaques pre-screened negative for SIV, simian T-cell leukemia virus, and simian type D retrovirus. For sampling, macaques were sedated with 10 mg/kg ketamine; before terminal sampling, animals were euthanized by an overdose of sodium pentobarbital. All procedures were approved by the Johns Hopkins University Institutional Animal Care and Use Committee and were conducted in accordance with guidelines set forth in the Animal Welfare Regulations and the Guide for the Care and Use of Laboratory Animals.

Circulating Platelet and Leukocyte Counts and Mean Platelet Volume
Citrated whole blood samples were used for platelet, lymphocyte, and monocyte counts and for determination of mean platelet volume (IDEXX, Westbrook, ME). The number of CD4+ lymphocytes was calculated as described [23].

SIV Load
Viral RNA was extracted from plasma using the QIAamp Viral RNA Mini Kit and analyzed by quantitative reverse transcription polymerase chain reaction (PCR), using a Quantitech Probe kit (Qiagen, Valencia, CA). Primers targeting unspliced SIV gag RNA were as follows: forward, 5′-GTCTGGGTCA TCTGGTGACATTC-3′; reverse, 5′-CCTAGGTTGCTCTGCA CTATCGTTTG-3′; 5′-FAM-3′-Black Hole-labeled probe 5′-CTTCCTAGTTGGTTTCACTTTTCTCTTCT-3′. Reaction conditions were 45 cycles at 94°C for 15 seconds, 55°C for 15 seconds, and 60°C for 30 seconds.

Platelet RNA Content (Reticulation)
Citrated whole blood was collected from 6 SIV-infected and 5 control macaques 10 days after inoculation. Platelet-rich plasma was harvested through centrifugation at 1000 × g for 15 minutes and fixed 1:20 in 2% paraformaldehyde overnight. Fixed platelet-rich plasma was washed twice with phosphate-buffered saline (PBS) and then diluted 1:10 in 2 mM ethylenediaminetetraacetic acid (EDTA)–PBS containing 5 µg/mL thiazole orange. After 2 hours at room temperature, a BD FACSCaliber flow cytometer was used to quantify mean channel fluorescence.

Hepatic Thrombopoietin Transcription
Liver tissue was harvested at necropsy from 6 SIV-infected macaques 10 days after inoculation and from 3 controls. RNA was extracted with an RNasy Plus Mini Kit (Qiagen). A hepatic complementary DNA library was created using oligo(dT)12–18 primers and Superscript III reverse transcriptase (Invitrogen, Grand Island, NY). Quantitative PCR amplification of a 152-bp sequence spanning exons 3 and 4 of thrombopoietin was completed using the forward primer 5′-ATTGCTCCTCGTGTTGCTATGC-3′, the reverse primer 5′-AAGGGTTAAACCTCTGGGACA-3′, and the 5′-Hex/3′-Iowa black FQ-labeled probe 5′-AGTAAACTIONCTTGACTCCCAGTCT-3′. The Quantitect
using a BD LSRFortessa (Figure 3).

The number of megakaryocytes per square millimeter).

Bone Marrow Megakaryocyte Density
Bone marrow was harvested at necropsy from 6 SIV-infected macaques 10 days after inoculation and from 5 controls. Five-micrometer-thick sections of fixed paraffin-embedded tissue were stained with hematoxylin and eosin. Stereo Investigator software (MBF Bioscience, Williston, VT) was used to define and sample a 3.35-mm² region of interest in bone marrow, and megakaryocytes were identified by their distinctive large size and complex nuclei at 200 times the original magnification (Figure 3A). The number of megakaryocytes per field was normalized to the area of sampled bone marrow to obtain the megakaryocyte density (reported as the number of megakaryocytes per square millimeter).

Megakaryocyte Surface Thrombopoietin Receptor (CD110) Expression
Bone marrow was harvested from 3 live SIV-infected and 3 live control macaques 10 days after inoculation, using a sternum/iliac aspiration needle. Marrow was collected into a syringe containing a 1:10 volume of citrate-dextrose, 2.5 mM EDTA, and 2.2 μM PGE₁ (Sigma-Aldrich, St. Louis, MO). Marrow was diluted 1:10 in 37°C megakaryocyte PBS buffer containing 13.6 mM sodium citrate, 1 mM theophylline, 11 mM glucose, 2.2 μM PGE₁, and 3% bovine serum albumin at pH 7.3 and 295 mOsm/L. Tissue was filtered through a 100-µM mesh, and cells were isolated through centrifugation for 10 minutes at 400 x g. Cells were resuspended to 10⁷ cells/mL, stained with anti-CD42a fluorescein isothiocyanate (FITC), and anti-CD110 phycoerythrin (PE) for 15 minutes and then fixed with 2% paraformaldehyde for 30 minutes prior to flow cytometry, using a BD LSRRFortessa (Figure 3D). Isotype controls (anti-IgG₁-FITC and anti-IgG₂b-PE) were used to set mean channel fluorescence gates, and 300 000 cells were counted per animal. All antibodies were from BD Biosciences.

Platelet Decline Is Transient During Acute SIV Infection
To define the nature of the decrease in platelet count during acute SIV infection, platelet counts were measured at 3
Platelet Production Is Maintained During Acute SIV Infection

Evidence for a change in platelet production has been inconclusive in the context of HIV infection, with increases, decreases, and no change reported [8, 10, 11, 25]. We examined whether a decrease in platelet production contributed to the decline in platelet count during acute SIV infection. With decline, compensatory platelet production normally increases, and platelets are released from bone marrow at a faster rate [26]. These platelets are larger and have an increased RNA content compared with more mature platelets as reflected by both increased mean platelet volume and increased reticulocytosis [26, 27]. SIV-infected macaques had significantly larger platelets (Figure 2A) and demonstrated a trend for increased reticulocytosis (Figure 2B), demonstrating a retained ability to mount a regenerative response to the decline in platelet count during acute infection.

The production of platelets by megakaryocytes is primarily maintained through the action of thrombopoietin binding to thrombopoietin receptor (c-MPL/CD110), which stimulates maturation of bone marrow megakaryocytes [28]. We therefore counted megakaryocyte numbers but detected no significant change in megakaryocyte density between infected and uninfected macaques (Figure 3B). Additionally, hepatic expression of thrombopoietin messenger RNA (mRNA) was upregulated (Figure 3C), with a trend toward increased expression of CD110 on megakaryocytes (Figure 3E), indicating that components necessary for an adequate thrombopoietic response remained intact during acute SIV infection.

Platelet Autoantibodies Are Not Detected During Acute SIV Infection

Homology between surface markers on the lentiviral envelope and platelet surface glycoproteins may result in the formation of platelet autoantibodies that contribute to the decline in platelet count by targeting platelets for destruction [12, 13]. We examined whether platelet autoantibodies were present during acute infection, using SIV-infected macaques from asymptomatic time points as positive controls. Platelet autoantibodies were not detected on day 10 in any of the 6 macaques examined, although autoantibodies directed against GPIIb/IIIa developed by day 42 during asymptomatic infection in 4 of 9 macaques. Platelet autoantibodies were not detected in mock-inoculated controls on day 10 or 42 after inoculation. These
observations make antibody-mediated platelet destruction an unlikely etiology for the decline in platelet count during acute infection.

Platelets Are Activated During Acute SIV Infection
Activated platelets are reported to participate in innate immune responses to many pathogens [4, 14, 21, 29], but platelet activation has yet to be documented during acute lentivirus infection. Activated platelets express several surface markers, including active conformation of GPIIb/IIIa (PAC-1 antibody binding) [30], the α-granule proteins P-selectin and CD40L [31, 32], and major histocompatibility complex class I (HLA-ABC) [33]. To determine whether platelet activation occurred during acute SIV infection, we first examined platelets in platelet-rich plasma for the surface activation marker P-selectin in SIV-infected macaques and controls (Figure 4A), and found statistically significant

Figure 2. Platelet production is maintained during acute simian immunodeficiency virus (SIV) infection. A, Mean platelet volume was significantly larger in SIV-infected macaques, compared with uninfected controls (P = .017 by the Mann–Whitney test). B, Thiazole orange mean channel fluorescence normalized to unstained fixed platelets, showing that the platelets from SIV-infected macaques also demonstrated increased RNA content, compared with uninfected controls (P = .052 by the Mann–Whitney test). Bars represent median values. *P < .05.

Figure 3. Adequate megakaryocyte numbers and increased thrombopoietin production available to support adequate platelet production. A, Representative section of bone marrow, stained by hematoxylin and eosin. Megakaryocytes (arrows) were identified by their large size and complex nuclei (original magnification ×200). The bar represents 10 μm. B, Simian immunodeficiency virus (SIV)–infected macaques demonstrated no significant change in megakaryocyte density, compared with uninfected controls (P = .33 by the Mann–Whitney test). C, SIV-infected macaques produce more hepatic thrombopoietin messenger RNA (mRNA), compared with uninfected controls (P = .048 by the Mann–Whitney test). D, Gating scheme for the quantification of surface CD110 expression on megakaryocytes from bone marrow. The example shown is from an uninfected macaque but is representative regardless of infection status. The shaded histogram represents CD42a+ megakaryocytes stained with anti-mouse IgG1x PE isotype control, the thin line represents CD42a+ non-megakaryocyte bone marrow cells, and the thick line represents CD42a+ megakaryocytes. E, CD110 expression on megakaryocytes from SIV-infected macaques trended higher than that for uninfected controls (P = .10 by the Mann–Whitney test). Bars represent median values. C(t), threshold cycle; Abbreviations: FITC, fluorescein isothiocyanate; FSC, forward scatter; PE, phycoerythrin; SSC, side scatter. *P < .05.
increases in both the percentage of platelets expressing P-selectin and the surface P-selectin expression level (Figure 4B), demonstrating platelet activation. Since processing steps used to prepare platelet-rich plasma, such as centrifugation, have the potential to induce exogenous platelet activation [34], we verified these results by measuring additional activation markers in minimally processed whole blood within 30 minutes of collection (Figure 5A). Significantly increased platelet surface expression of 1 alternative activation marker measured (CD40L) and trends toward increased expression of 2 other activation markers (P-selectin and HLA-ABC) on day 10 after infection provided further evidence for platelet activation during acute lentivirus infection (Figure 5B).

Platelets Are Sequestered in Platelet-Monocyte Interactions During Acute SIV Infection
The percentage of P-selectin–expressing activated platelets in platelet-rich plasma was inversely correlated with extent of the decline in platelet count (\(r_s = -0.72; P = .023\)), indicating that increased platelet activation corresponded with the magnitude of the decline in platelet count. Activated platelets can physically interact with circulating white blood cells to form platelet-leukocyte aggregates [33, 35], and such interactions effectively remove free platelets from circulation. To determine whether an increase in the number of platelet-monocyte or platelet-lymphocyte aggregates developed during acute SIV infection, flow cytometry was used to identify these aggregates (Figure 6A and Supplementary Figure 1A). We observed a trend toward increased numbers of CD42a⁺ platelet-monocyte aggregates during acute SIV infection (totaling 42%–78% of monocytes bound, or 485–1391 platelet-monocyte aggregates/µL; Figure 6B). In contrast, the number of circulating platelet-lymphocyte aggregates was lower (totaling 0.2%–1.6% of lymphocytes bound, or 4–37 lymphocytes bound to platelets/µL; Figure 6B and Supplementary Figure 1B).

Monocytes can be divided into 3 distinct subsets: CD14\(^{\text{high}}\) CD16⁻ classical monocytes progress through a CD14\(^{\text{high}}\)CD16\(^{\text{+}}\) intermediate phenotype to give rise to CD14\(^{\text{low}}\)CD16\(^{\text{+}}\) nonclassic monocytes [36]. When these subsets were evaluated individually with respect to platelet binding, the latter 2 subsets demonstrated significantly increased platelet-monocyte aggregate formation during acute SIV infection, with 83%–97% of CD14\(^{\text{high}}\)CD16\(^{\text{+}}\) and 60%–95% of CD14\(^{\text{low}}\)CD16\(^{\text{+}}\) monocytes in platelet-monocyte aggregates (Figure 6D). Furthermore, the number of CD16\(^{\text{+}}\) platelet-monocyte aggregates correlated with the magnitude of the decline in platelet count in acute infection (Figure 6C).

DISCUSSION
These studies in the SIV/macaque model demonstrate that circulating platelets become activated and form aggregates with
monocytes during acute SIV infection, corresponding with a decrease in circulating platelet numbers. We did not find evidence for either decreased platelet production or increased antibody-mediated platelet destruction during acute infection. The monocytosis that accompanies the decline in platelet count during acute HIV/SIV infection increases the number of circulating monocytes available to bind free platelets [37], thereby sequestering them in platelet-monocyte aggregates and contributing to the decline in platelet count. The ultimate fate of these platelet-monocyte aggregates warrants future study, and, given the majority of CD16+ monocytes in circulation are complexed with platelets, platelet-monocyte interactions may play a pivotal role in HIV disease progression.

In our SIV studies, the increase in the percentage of monocytes bound to platelets during acute infection was especially marked in CD16+ monocytes, with the majority of CD14^{high} and CD14^{low}CD16+ monocytes bound to platelets. This finding complements a recent report documenting CD16+ platelet-monocyte aggregates in asymptomatic HIV-infected humans [16] and is especially significant, given potential roles for CD16+ monocytes in the pathogenesis of HIV infection. CD16+ monocytes are more likely than their CD16– counterparts to be productively infected with HIV-1 [38]. Furthermore, CD16+ monocytes more readily traverse resting endothelium in a culture system [39] and also have been observed to cross the blood-brain barrier during acute infection in SIV-infected macaques [29], demonstrating that CD16+ monocytes have the potential to transport HIV into organs to establish latent viral reservoirs.

The number of CD16+ monocytes bound to platelets correlated with the magnitude of the decline in platelet count. Platelets associate with monocytes via P-selectin–PSGL-1 interactions [40];
downstream signaling from PSGL-1 results in the activation of transcription factors that can alter monocyte/macrophage gene expression profiles [41]. Incubation of activated platelets with unstimulated CD14highCD16– monocytes results in a shift of monocyte phenotype to CD16+ [42], and platelet-monocyte aggregates demonstrate increased integrin expression and readily adhere to endothelium, compared with unbound monocytes [43].

Given the correlation between the decline in platelet count and the number of CD16+ platelet-monocyte aggregates, the large percentage of CD16+ monocytes involved in platelet-monocyte aggregates during acute infection, and the knowledge that incubating CD16+ monocytes with activated platelets results in a phenotype switch [16], activated platelets may drive the adoption of the CD16+ phenotype by monocytes in HIV and therefore directly contribute to the pathogenesis of lentivirus infection.

We examined platelet activation status by measuring platelet surface markers, including P-selectin, CD40L, and HLA-ABC. Our data complement findings from previous studies that identified increased platelet P-selectin or CD63 expression in HIV-infected individuals at asymptomatic and terminal stages of infection [15–17]. It remains to be determined whether platelet...
activation during acute SIV infection is specific to lentivirus infection or represents a general response to acute inflammatory stimuli. Platelets can be activated in vitro directly by viral components such as HIV tat [44], but there is no evidence to suggest that HIV is required for the platelet activation. Platelets are participants in innate immune responses [14] and may be activated by multiple cytokines, including tumor necrosis factor α and interleukin 6, that have elevated levels during acute lentivirus infection [4, 45–47]. Ongoing stimulation by these cytokines throughout acute infection has the potential to maintain the diminishing pool of platelets in a state of activation, even as the activated platelets are selectively sequestered in platelet-monocyte aggregates. As signaling events downstream of viral-sensing Toll-like receptors 7, 8, and 9 regulate the expression of these cytokines during acute infection [48], platelet activation could result from the innate immune response to a number of viruses. Platelet activation has been observed during acute cytomegalovirus infection [49], during acute dengue [50], and after vaccination against influenza [42]. The mechanisms leading to platelet activation during many acute viral infections still need to be defined.

Our studies demonstrated that the ability to produce platelets is not impaired during acute SIV infection. Increased platelet size and reticulation, coupled with increased thrombopoietin transcription, normal to increased megakaryocyte numbers, and increased megakaryocyte expression of CD110, all support an intact regenerative response to the decline in platelet count. Decreased platelet production may contribute to the decline in platelet count during asymptomatic infection, and this may explain previous studies that have presented evidence for decreased thrombopoiesis [8, 10, 25]. Similarly, although we detected no platelet autoantibodies during acute SIV infection, antibody-mediated platelet destruction may play a role during later stages of disease. For example, we detected anti-GPIIb/IIIa antibodies in 4 of 9 SIV-infected macaques during asymptomatic infection, consistent with reports of platelet autoantibodies in some but not all HIV-infected individuals [8].

By focusing on acute infection in the SIV/macaque model of HIV, we were able to demonstrate that platelet activation and sequestration in platelet-monocyte aggregates, rather than decreased platelet production or increased antibody-mediated destruction, contributed to the nadir of the decline in platelet count. Since acute infection is critical in the pathogenesis of lentivirus infection, interactions between platelets and CD16+ monocytes may reflect platelets’ participation in the innate immune response. As the mechanisms underlying the decline in platelet count likely vary at different stages of infection, dissecting out the specific contributory factors at time points following acute infection, including both asymptomatic and terminal stages, will be facilitated by additional SIV-based studies.

**Supplementary Data**

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

**Notes**

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