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Detection of Circulating Platelet-Monocyte Complexes in persons infected with Human Immunodeficiency Virus Type-1

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Abstract

Activated platelets form transient aggregates with monocytes in circulation and have a half-life of approximately 30–60 minutes. These complexes are increased in various inflammatory conditions and are an early marker of myocardial infarction. HIV-1 infection is associated with chronic inflammation, and increased CD16⁺ inflammatory monocytes have been observed in these individuals, probably as a result of increased interaction with platelets. However, narrow detection period and platelet activation during sample processing pose significant problems in detecting platelet-monocyte complexes (PMCs). A method was standardized addressing these difficulties, to enumerate PMCs involving CD16⁺ or CD16[−] monocytes in whole blood using flow cytometry. Blood collected from healthy individuals was treated with either collagen (for platelet activation) or LPS (for monocyte activation) and subsequently used to study effect of these treatments on PMC formation. This method was also validated for the *ex vivo* quantitation of PMCs in blood obtained from persons infected with HIV. The *in vitro* results demonstrated that platelet activation, but not monocyte activation, resulted in significant increase in PMC formation. There was a significant increase in CD16⁺ PMCs and platelet activation, in samples obtained from persons infected with HIV as compared to those without HIV infection. Furthermore, PMC percentages correlated positively with platelet activation. These findings improve the ability to detect PMCs and shed light on HIV pathogenesis.

1. Introduction

Platelets, small anucleated cells, are a lead player in thrombosis, and are critical not only in preventing excessive loss of blood, but also in the recruitment of inflammatory cells such as monocytes and neutrophils to the site of injury. Under normal physiologic conditions, platelets reside in an inactive form; however they are quickly and easily activated at sites of vascular damage. Platelets can also become activated in response to pro-inflammatory cytokines or infective agents (Semple and Freedman, 2010) as well as by shear force (Kroll et al., 1996). Platelet activation by all of these stimuli, even in the absence of any detectable vessel damage, has opened up a new prospect in platelet function, i.e. inflammation and

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immune regulation. Just as the capacity of activated platelets to form aggregates with other platelets is crucial for their thrombogenic function, their pro-inflammatory activity is mediated through interactions with other leukocytes in circulation, and the subsequent release of cytokines and chemokines thus facilitates inflammation (Freedman and Loscalzo, 2002). Interestingly, monocytes have a competitive advantage over other leukocytes in forming complexes with platelets, and these circulating platelet monocyte complexes (PMCs) are considered a more sensitive marker of platelet activation than P-selectin expression (Michelson et al., 2001). Increased PMCs have been demonstrated in Alzheimer's disease (Sevush et al., 1998), myeloproliferative disorders (Villmow et al., 2002), autoimmune disorders (Joseph et al., 2001), and in individuals with cardiovascular risk factors (Gkaliagkousi et al., 2009). However, these complexes are known to have a very short half-life *in vivo*. Intravenous injection of activated platelets led to sequestration of leukocytes in apoE^{-/-} mice within 5 minutes, an effect that lasted up to 180 minutes in the case of monocytes (Rinder et al., 1991). Accordingly, another study in baboons revealed similar results where these complexes were undetectable at 2 hours after injection of activated platelets (Michelson et al., 2001).

The interaction between platelets and monocytes is predominantly facilitated through binding of P-selectin on platelets with P-selectin glycoprotein ligand-1 (PSGL-1) on monocytes, and is considered to be dependent on divalent cations (Bournazos et al., 2008; Fernandes et al., 2003). It has also been demonstrated that the cross-talk with activated platelets induces monocytes to mature into a more pro-inflammatory subtype (Bouchon et al., 2000; Weyrich et al., 1996; Weyrich et al., 1995). Hence, enumeration of these complexes in whole blood might be indicative of the immune-inflammatory status in diseases associated with chronic inflammation, for example, in the context of Human Immunodeficiency Virus Type-1 (HIV-1; henceforth referred to as HIV) infection.

Flow cytometry is the most widely used method of PMC detection, where PMCs are defined as the monocytes expressing platelet markers. However, detection of these complexes is not very straightforward due, in part, to the fact that platelets can undergo spontaneous activation during sample processing and once activated, they can immediately form complexes with surrounding monocytes. These aggregates, formed as byproducts of the experimental procedure, can dilute the detection of already existing complexes. Even though many groups have reported the association of these complexes with various diseases using flow cytometry, not many have considered these difficulties while processing the samples (Gkaliagkousi et al., 2009; Joseph et al., 2001; Ogura et al., 2001; Passacquale et al., 2011; Sevush et al., 1998). Therefore, it was proposed to modify the current, reported methods in order to eliminate complexes that can be induced experimentally, while preserving the resident PMCs. The method was also verified for its efficacy to quantitate PMCs *in vitro*, by studying the effect of platelet and monocyte activating agents on the interaction of these two cell types in cell culture, and *ex vivo*, in blood collected from persons with or without HIV infection. The results demonstrate that by fixing the blood prior to staining, it is possible to preserve the PMCs efficiently, while limiting consequential platelet activation. Thus, this method is well suited for use in detecting these inflammatory complexes for a broad range of applications.

2. Materials and Methods

2.1. Reagents and antibodies

Anti-CD14 PE, anti-CD16 PE Cy7, anti-CD62P FITC antibodies and compensation beads were purchased from BD Biosciences, CA, USA. Anti-CD61 AF 647 and collagen were purchased from AbD Serotec, USA and Chronolog, PA, USA respectively.

Paraformaldehyde (PFA) and lipopolysaccharide (LPS) were obtained from Sigma Aldrich, MO, USA. ACK RBC lysis buffer was purchased from Invitrogen, CA, USA.

2.2. Study patients

Persons with (N=8) and without (N=9) HIV infection (without any occurrence of cardiovascular disease at least one preceding year) were enrolled in the study. Written informed consent was obtained from all patients according to University of Rochester Research Subjects Review Board guidelines. Blood samples were drawn in ACD (acid citrate dextrose) buffered vacutainers (BD Biosciences, CA, USA). All persons with HIV infection were on antiretroviral therapy (ART) at the time of the draw.

2.3. Staining method for identification of PMCs

100 μ l blood was processed in any of the following four ways: stain-lyse-wash-fix (SLWF), stain-fix-lyse-wash (SFLW), fix-lyse-wash-stain (FLWS), or fix-wash-lyse-wash-stain (FWLWS) in an effort to optimize the staining procedure for detection of PMCs using flow cytometry. FWLWS was selected as the staining method of interest and all subsequent samples were processed using that method. Briefly, 100 μ l blood was fixed with 4% PFA for 15 minutes in the dark at room temperature. After fixing, the blood sample was washed twice with 1 ml staining buffer (1 $\times$ PBS containing 2% BSA) by centrifugation at $0.4 \times g$ for 5 minutes. Red blood cells (RBCs) were then lysed using ACK lysis buffer for 5 minutes at room temperature in the dark, and the remaining cells were washed twice as above. Cells were re-suspended in 100 μ l staining buffer and stained with various antibodies, 10 μ l CD14, 3 μ l CD16, 10 μ l CD62P and 3 μ l CD61, for 25 minutes in the dark at room temperature. The samples were then washed twice, re-suspended in 250 μ l staining buffer and were acquired on the flow cytometer (Accuri C6). Unstained cells and cells stained with only CD14 and CD16 were used as controls. 50,000 events per tube were acquired in the “Leukocyte” gate at the same instrument settings for quantitation of PMCs. The same tubes were also acquired with 10,000 events in the “Cells” gate to measure platelet percentages and activation in terms of CD62P expression.

2.4. Gating strategy used for quantitation of PMCs

For each sample, unstained cells were used to define the “Leukocyte” gate on a forward and side scatter chart (FSC/SSC). Cells in the leukocyte gate were used to formulate the next chart and subsequently, the monocytes were gated based on their FSC/SSC characteristics. These two gates were then applied to the cells stained with antibodies against CD14 and CD16 only. These cells were used to differentiate between two monocyte subtypes, CD14⁺CD16⁻ and CD14⁺CD16⁺, henceforth referred to as CD16⁻ and CD16⁺ monocytes. Furthermore, the same cells were used to define the gate for detecting PMCs, i.e. monocytes expressing CD61, and were labeled as CD16⁻CD61⁺ and CD16⁺CD61⁺. Each of these gates was then applied to the cells stained with all four antibodies to calculate percentages of CD16⁻ and CD16⁺ PMCs in the total monocyte population.

2.5. Quantitation of platelet percentages and activation

Unstained cells were used to define the gate comprised of platelets and leukocytes on a FSC/SSC plot (labeled as “Cells” gate). Sizing beads (Mega Mix, Biocytex, Marseille, France) were also used to define the “platelets” gate (0.9–3 μ m) and to eliminate debris as well as microparticles. The cells stained with CD14 and CD16 antibodies alone were then used to define the CD61⁺ and CD62P⁺ gates, and these gates were then applied to the cells stained with anti-CD61 and anti-CD62P to calculate the percentage of CD62P⁺ platelets and CD62P expression as Median Fluorescence Intensity (MFI).

2.6. Quantitation of PMCs after *in vitro* platelet and monocyte activation and in persons with and without HIV infection

For the *in vitro* treatments, 100 μ l blood per tube was treated with either 2 μ g/ml collagen or 10 ng/ml LPS, or alternatively was left untreated and was incubated at 37°C in a 5% CO₂ incubator for 45 minutes (N=4). After the treatment, blood was fixed, stained, and acquired as detailed above. Blood samples obtained from persons with (N=8) or without (N=9) HIV infection were processed as above for enumeration of PMCs.

2.7. Tumor necrosis factor alpha (TNF α) ELISA

Monocytes were isolated from peripheral blood mononuclear cells (PBMCs) derived from a healthy donor using a MACS CD14⁺ monocyte isolation kit (Miltenyi Biotech Inc., CA, USA). Monocytes (0.5×10^6) were treated in triplicate with 10 ng/ml of LPS or left untreated. After 8 hours, 100 μ l supernatant was collected and used to measure TNF α levels using a TNF α ELISA kit (Invitrogen, CA, USA; sensitivity >1.7 pg/ml).

2.8. Statistical analysis

Statistical analysis was performed using Graph Pad Prism 4.0 software. Comparison between PMC percentages, CD62P MFI, and percent CD62P⁺ platelets in LPS or collagen treated samples, as well as comparison between the same populations in samples obtained from persons with or without HIV infection was done by Mann-Whitney U test. Correlation between percent CD16⁺CD61⁺ monocytes and CD62P MFI was analyzed by the Spearman correlation test.

3. Results

3.1. Improved staining method for detection of PMCs in whole blood

A routine method of whole blood sample preparation for analysis by flow cytometry involves staining with fluorochrome-tagged antibodies of interest, followed by RBC lysis, washing, and finally fixation, which is optional (stain-lyse-wash-fix; SLWF; Fig. 1A). However, this method could not be used to detect PMCs because, first, these complexes have a short half-life and would likely be disrupted during sample processing and, second, the staining method and centrifugation steps have the potential to activate platelets and may result in *de novo* formation of complexes. In order to overcome these difficulties, fixing the samples as early as possible during sample processing is essential. Consequently, three combinations of the different steps involved in the staining procedure were tested as explained previously, i.e. stain, lyse, wash and fix. Of these, the stain-fix-wash-lyse (SFLW) combination resulted in incomplete RBC lysis and indistinct cluster formation on FSC/SSC plots, as well as non-specific staining (Fig. 1B). Additions of fixative in the presence of excess unbound antibodies likely led to non-specific staining and hence, the fix-lyse-wash-stain (FLWS) method was tested next. Although non-specific staining was reduced when following this method, it still caused incomplete RBC lysis and indistinct cluster formation (Fig. 1C), which led to difficulties in gating the leukocyte and monocyte clusters when performing analysis. Prolonged exposure of cells to PFA throughout the staining protocol seemed to be the underlying cause of these problems and therefore, an additional wash step was introduced following fixation in an effort to remove the fixative (fix-wash-lyse-wash-stain; FWLWS; Fig. 1D). Addition of the extra wash step resulted in complete RBC lysis and well-defined cell clusters and thus, all data showed henceforth was obtained using this method. The gating strategy used to calculate PMC percentages and platelet activation, in terms of CD62P expression, is shown in Figures 2 and 3, and described in detail in sections 2.4 and 2.5 respectively.

3.2. Increase in PMC formation following platelet activation

Whole blood obtained from healthy HIV negative donors was treated with either LPS, to activate monocytes, or with collagen, to activate platelets, and subsequently used to enumerate PMCs. Secretion of TNF α by monocytes was measured as a marker of monocyte activation, and LPS treated monocytes showed increased levels of TNF α as compared to non-treated monocytes ($p=0.002$; Fig. 4A). Additionally, there was a significant increase in platelet activation as indicated by an increase in MFI of CD62P expression (Fig. 4B) and by the percentage of CD62P $^{+}$ platelets (Fig. 4C) in whole blood treated with collagen as compared to non-treated ($p=0.0082$) or LPS treated ($p=0.0095$) blood. Blood samples treated with collagen also displayed higher levels of PMCs in CD16 $^{+}$ (Fig. 4D) and CD16 $^{-}$ (Fig. 4E) monocytes as compared to non-treated and LPS treated samples ($p < 0.05$).

3.3. Increased levels of CD16 $^{+}$ PMCs in samples obtained from persons with HIV infection correlated positively with activated platelet numbers

As compared to samples obtained from persons without HIV infection, samples obtained from persons with HIV infection contained significantly increased percentages of CD16 $^{+}$ CD61 $^{+}$ monocytes (CD16 $^{+}$ PMCs; $p=0.0079$; Fig. 5A). While the CD16 $^{-}$ CD61 $^{+}$ monocyte percentages were also higher as compared to persons without HIV infection, these were not statistically significant (Fig. 5B). Platelets within the samples obtained from persons with HIV infection expressed more CD62P ($p=0.029$; Fig. 5C) as compared to those without HIV infection, indicating increased activation and CD62P values correlated positively with CD16 $^{+}$ PMC percentages ($p < 0.05$; Fig. 5D).

4. Discussion

Chronic immune activation associated with increased inflammation is one of the hallmarks of HIV infection and is observed even in the acute phase of disease progression. Monocytes contribute substantially toward HIV pathogenesis, both by secreting various pro-inflammatory cytokines and chemokines upon activation and by acting as a reservoir of latent HIV infection (Fischer-Smith et al., 2001; Williams and Hickey, 2002). Monocytes can be activated upon encounter with bacterial endotoxins, pro-inflammatory cytokines, and other cells of the immune system, and also upon interaction with activated platelets. Previous studies from our laboratory, and from those of others have shown that despite ongoing thrombocytopenia, an increased number of activated platelets, as well as markers of platelet activation such as soluble CD40L (sCD40L) (Davidson et al., 2011) and platelet activating factor (PAF) (Gelbard et al., 1994), are found in persons with HIV infection. sCD40L increases the permeability of the blood brain barrier to virus thereby potentially allowing more monocytes to enter the central nervous system (CNS; Davidson et al, unpublished data). On a related subject, a study by Ragin et al, on older patients from the MACS cohort (an advanced HIV infection cohort), has shown that platelet decline was found to be associated with reduced gray matter volume and increased risk of dementia (Ragin et al., 2011). In addition to this, there is growing evidence that the cross-talk between monocytes and activated platelets promotes the activation and modulation of monocyte behavior, and that these interactions at sites of injury and infection may function to further promote the inflammatory response [(Bournazos et al., 2008) and reviewed by Stephen et al (Stephen and Dransfield, 2010)]. Hence, it is plausible that in persons infected with HIV, monocyte activation via platelet-monocyte cross-talk could result in enhanced migration of CD16 $^{+}$ monocytes across the blood brain barrier and not only spread the virus to the CNS compartment (if they are latently infected), but also exacerbate the CNS associated inflammatory disorders in these patients. Indeed, studies have shown that there are increased CD16 $^{+}$ CD163 $^{+}$ cells in the CNS of individuals with HIVE (HIV-associated encephalitis) [(Fischer-Smith et al., 2001) and reviewed in (Fischer-Smith et al., 2008)]. Due to the

complex interplay between platelets and monocytes, both alone and together, during immune pathogenesis of HIV infection, it is essential to devise a better methodology to detect PMCs. With this goal in mind, a method to quantitate PMCs in whole blood was standardized and was subsequently employed to assess the percentages of circulating PMCs in blood collected from persons infected with HIV.

Although several previous reports have enumerated PMCs using flow cytometry in regard to various disease conditions, the methodologies used thus far have failed to consider spontaneous platelet activation and the resulting consequence on *de novo* PMC formation (Gkaliagkousi et al., 2009; Joseph et al., 2001; Ogura et al., 2001; Passacuale et al., 2011; Sevush et al., 1998). Thus, in the efforts to formulate an optimized method for detection of PMCs, fixing the blood samples immediately following collection was given prime importance, as it helped to conserve PMCs and avoid experimental artifacts. Demonstrated herein, is a fix-wash-lyse-wash-stain method to fluorescently label blood samples, which when used along with a progressive gating strategy and FMO (fluorescence minus one) controls, allows for efficient enumeration of PMCs. The additional wash step performed after sample fixation was necessary to avoid the toxicity caused by excessive exposure to PFA.

One significant concern in adopting this method was that the fixing of samples prior to staining might alter the antibody binding capacity to some extent; however this was deemed a reasonable trade-off given the advantages of this method as outlined above. Nonetheless, *in vitro* whole blood treatments with LPS and collagen were performed, to serve as monocyte and platelet activating reagents, respectively, in order to ensure that it is possible to capture the changes in PMC percentages induced by these treatments using the method of detection described here. Results indicated that platelet-monocyte interaction increased significantly upon platelet activation as compared to monocyte activation. These results corroborate an earlier report by Rinder *et al.* who showed that platelet-leukocyte complexes increased upon platelet activation and that this interaction was dependent on monocyte activation only to a very limited extent (Rinder et al., 1991).

Upon validation of the procedure, persons with or without HIV infection (without any incidence of cardiovascular disease at least one year prior to enrollment in the study) were enrolled to assess whether HIV infection had any effect on platelet-monocyte interactions. All of the individuals enrolled in the study were on ART and had low viral load with CD4⁺ T cell count above 500 cells/mm³ (data not shown). The results show that despite the viral suppression induced by successful ART, the number of circulating PMCs was significantly higher in the inflammatory monocyte subset (i.e. CD16⁺ monocytes) of these individuals as compared to healthy individuals without HIV infection, and correlated positively with the extent of platelet activation. The PMC percentages in the classical CD16⁻ monocyte subset were also higher in samples obtained from persons infected with HIV, although the effects were not statistically different from controls. Consistently, our data (not shown) and previous reports (Pulliam et al., 1997; Thieblemont et al., 1995) demonstrate a significant increase in CD16⁺ monocyte percentages in samples obtained from persons infected with HIV.

This study defines a flow cytometric method to quantify PMCs in clinical specimens. The findings suggest that persons infected with HIV have increased levels of platelet-monocyte complexes, particularly within the inflammatory monocyte population despite successful ART. Furthermore, this data suggests that either platelets have an increased preference to associate with CD16⁺ monocytes, or that alternatively, the platelet-monocyte cross-talk causes maturation of classical monocytes to the CD16⁺ phenotype; thus warranting further investigation into whether this interaction enhances the monocyte capacity to cross the blood

brain barrier, as well as the role of these monocytes in HIV associated neuro-cognitive impairment.

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Abbreviations

PMCs	platelet-monocyte complexes
HIV-1	Human Immunodeficiency Virus Type-1
PSGL-1	P-selectin glycoprotein ligand-1
PFA	paraformaldehyde
LPS	Lippopolysaccharide
ACD	acid citrate dextrose
ART	anti retroviral therapy
SLWF	stain lyse wash fix
SFLW	stain fix lyse wash
FLWS	fix lyse wash stain
FWLWS	fix wash lyse wash stain
MFI	median fluorescence intensity
TNFα	tumor necrosis factor alpha
RBCs	red blood cells
CNS	central nervous system
sCD40L	soluble CD40 ligand
PAF	platelet activating factor
PBMCs	peripheral blood mononuclear cells

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Highlights

1. Optimum method to quantify PMCs in whole blood using flow cytometry
2. A method to preserve PMCs while preventing experimental artifacts during processing
3. Platelet activation, not monocyte activation leads to increase in PMC formation.
4. In HIV infected individuals CD16⁺ PMCs are increased compared to healthy controls.
5. CD16⁺ PMCs are positively correlated to platelet activation in these individuals.

Figure 1. Comparison between staining methods for detection of platelet-monocyte complexes in whole blood using flow cytometry

100 μ l whole blood was processed by one of four methods [i.e. stained, lysed, fixed, washed (SLFW); stained, fixed, lysed, washed (SFLW); fixed, lysed, washed and stained (FLWS); or fixed, washed, lysed, washed, stained (FWLWS)] and acquired on a flow cytometer. The antibodies used were CD14, CD16, CD61 and CD62P. FWLWS was determined to be the optimum method for enumeration of PMCs.

Figure 2. Progressive gating strategy to quantitate platelet-monocyte complexes in whole blood 100 μ l blood was fixed with 4% PFA and washed, followed by RBC lysis and staining with antibodies against CD14, CD16, CD61 and CD62P for 25 minutes at room temperature. The stained cells were acquired on an Accuri C6 flow cytometer at the same instrument settings. (A) 50,000 events were acquired per sample in the “Leukocyte” gate. (B and C) Monocytes were gated and were classified as CD14⁺CD16⁻ and CD14⁺CD16⁺ cells. (D–G) PMCs were identified in both subsets of monocytes, as CD16⁺CD61⁺ and CD16⁻CD61⁺ cells. Cells stained with only CD14 and CD16 were used as control to identify monocytes expressing CD61.

Figure 3. Flow cytometric analysis of platelet percentages and activation

100 μ l blood was fixed and stained as in Figure 2. The stained cells were acquired on Accuri C6 flow cytometer at the same instrument settings. (A) 10,000 events were acquired per sample in the “Cells” gate. (B) Platelets were gated using sizing beads (0.9 – 3 μ m) and subsequently quantified as the percentage of total cell population as defined in (A) (C) Unstained cells were used to define the “CD62P⁺” gate (D and E) Platelet activation was measured as percentage and MFI of CD62P expression.

Figure 4. Assessment of PMC formation upon *in vitro* treatments to activate platelets and monocytes in whole blood

(A) Monocytes were isolated from PBMCs using CD14 $^{+}$ magnetic beads and were treated with LPS (10 ng/ml) or alternatively left untreated. Measurement of TNF α (in supernatant) as a marker of monocyte activation indicated a significant increase in response to LPS. (B–E) 100 μ l whole blood was treated with either LPS (10 ng/ml) or collagen (2 μ g/ml) for 45 minutes, or left untreated. Treated samples were then fixed, stained, and acquired as described previously. (B and C) Expression of CD62P, in terms of MFI and percent CD62P positive platelets, was significantly increased in collagen treated blood as compared to non-treated or LPS treated blood. (D and E) Blood treated with collagen displayed a significant increase in the percentages of CD16 $^{+}$ and CD16 $^{-}$ PMCs, as compared to non-treated or LPS treated blood.

Figure 5. Enumeration of PMCs in samples obtained from persons with or without HIV infection Blood samples were collected from persons with (N=8) or without (N=9) HIV infection. 100 μ l blood per tube was stained as described previously and used for acquisition on an Accuri C6 flow cytometer. Unstained, as well as cells that had been stained with anti-CD14 and anti-CD16 alone, were used as controls. **(A)** Percent CD16⁺ PMCs were significantly increased in HIV infected samples as compared to uninfected samples. **(B)** Increase in CD16⁻ PMCs in HIV infected samples was not statistically significant. **(C and D)** Platelet CD62P expression was significantly higher in HIV infected samples, as compared to uninfected samples, and correlated positively with CD16⁺ PMCs.