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A FLOW CYTOMETRY METHOD FOR CHARACTERIZING PLATELET ACTIVATION

Brian Alzua[1], Mark Smith[1], Yan Chen[1]

[1]American Preclinical Services, Minneapolis, MN

ABSTRACT

Hemocompatibility testing is critical for assessing the safety of blood-contacting medical devices. Comprehensive hemocompatibility testing requires examining a wide range of possible adverse effects cause by direct or indirect blood contact, such as hemolysis, complement activation, and thrombus formation [1]. Moreover, these domains each encompass complex intercellular processes with many potential targets for analysis. For example, the current testing paradigm of platelet function may involve exposing the device to human whole blood and performing simple blood counts and/or macroscopic evaluation to determine the extent of platelet activation and clot formation as described in ASTM F2888-19. However, this approach does not capture any observations for device-mediated initiation of any steps in the platelet activation pathway prior to aggregation. We have validated a method to evaluate platelet activation by quantifying surface p-selectin expression after exposure to various materials. This method will provide an additional level of detail about potential platelet activating properties of a medical device.

Flow cytometry has been used previously to measure platelet activation for clinical and research purposes. We sought to adapt this method to test for platelet activation induced by exposure of blood to medical devices or materials. We determined that processing fresh whole blood to platelet-rich plasma (PRP) by gentle centrifugation enhanced the signal compared to fresh blood itself. In each experiment, devices were exposed to PRP according to an extraction ratio of 6 cm^2/mL for 1 hour. A blank control consisting of untreated PRP, and a positive control consisting of ADP, a potent agonist, were also used. After the exposure, excess plasma was removed from the articles and combined with anti-CD61 (to stain for platelets) and anti-CD62P (to stain for activated platelets) antibodies. Flow cytometry was then performed to quantify the percentage of CD62P+ over the total CD61+ cells to measure the percentage of activated platelets. In order to optimize the method, we investigated the effect of several experimental factors, including anticoagulant usage, donor variability, and selection of reference materials to serve as controls. Our results indicate that the flow cytometry-based method is consistent and reproducible, quick and easy to perform, and is well-correlated with results from the standard platelet and leukocyte count assay. The flow cytometry-based platelet activation method is a powerful supplement to the standard regimen of medical device hemocompatibility testing.

Keywords: hemocompatibility, platelet activation, medical devices.

1. INTRODUCTION

Activation of platelets is an important initiating step in the process of thrombus formation. While current hemocompatibility medical device testing paradigms involve measuring thrombus formation and platelet counts after exposing devices to whole blood, upstream events, such as platelet activation, are generally not considered [1]. Examining the preliminary process of platelet activation caused by a medical device may provide valuable information about the specific mechanism by which a device interacts with blood. Furthermore, measuring platelet activation may be a more sensitive metric of hemocompatibility than simple platelet counts.

Flow cytometry is a powerful laboratory technique for measuring expression of cellular surface markers, and has been previously used to measure platelet activation. [1, 2]. This procedure involves use of fluorescent antibodies for CD61 (a marker of all platelets) and CD62P (also called p-selectin, expressed on activated platelets). We adapted this method to test for platelet activation induced by exposure of blood to medical devices or materials.

To tailor the flow-based platelet activation method for testing of medical devices, we first attempted to identify reference materials suitable to be used as negative and positive controls. Preliminary experiments revealed that processing of freshly

drawn human whole blood to platelet rich plasma (PRP) by gentle centrifugation yielded lower baseline activation in untreated platelets and a higher maximum activation in agonist-treated samples. Similar to existing methods, we exposed materials to PRP according to a surface/volume extraction ratio based on ISO 10993-12. These experiments identified several candidate materials for use as positive and negative controls. Next, we examined common experimental variables from existing hemocompatibility tests to determine their effect on the platelet activation assay. Tests for optimal anticoagulant selection revealed that sodium citrate was preferable to heparin based on high baseline activation levels in heparinized blood. Lastly, we examined the effect of using multiple donors, as eliminating the effects of donor variability is critical to standardizing a test for widespread or repeated use. These results form the foundation of a platelet activation assay for medical devices, simple to perform with low device requirement, and extensible through the addition of fluorescent markers for other platelet activation biomarkers.

2. METHODS

2.1 Blood Collection & Plasma Processing

Fresh human blood was collected by venipuncture from healthy human donors. Donors abstained from anticoagulating drugs for at least 7 days prior to the blood draw. Immediately after collection, blood was centrifuged at 200 x *g* for 20 minutes, with no deceleration, to separate platelet rich plasma (PRP). The upper, cloudy layer of plasma was removed from the collection tubes and pooled.

2.2 Sample Preparation

A flat sheet of High Density Polyethylene (HDPE) was obtained from Hatano (Kanegawa, Japan) and cut to into 1 cm x 1.5 cm strips. Glass Beads with a diameter of 3 mm were obtained from Sigma Aldrich (St. Louis, MO); the surface area of each bead was calculated, a number of beads was counted and placed into a polypropylene tube, and the total surface area was recorded. Black rubber stoppers were obtained from Fisher Scientific (Hampton, NH), cut into discs and measured to calculate the total surface area. Latex tourniquets were obtained from Hygenic (Akron, OH) and cut into 1 cm x 1.5 cm strips. The total surface area of each prepared control was recorded and the volume of PRP required to be added to each tube was calculated based on an extraction ratio of 6 cm^2 /mL. Prepared materials were placed into appropriately sized polypropylene tubes in triplicate.

2.3 Exposure

An appropriate volume of PRP was added to each sample. A positive control consisting of an equivalent volume of PRP spiked with 50 µM adenosine diphosphate (ADP) in phosphate buffered saline (PBS) (Sigma-Aldrich), and a negative control consisting of PRP mixed with PBS, were also prepared. Sample and control tubes were incubated at room temperature for the desired exposure time.

2.4 Flow Staining

Anti-CD61:PerCP-Cy5.5 (Clone VI-PL2) and anti-CD62P:PE (Clone AK4) antibodies were purchased from Becton Dickinson (Franklin Lakes, NJ). Anti-CD61 and anti-CD62P were diluted in phosphate buffered saline to prepare a master staining mix for each sample. 50 µL of exposed plasma was transferred from each sample and control tube to new flow cytometry tubes; 50 µL of staining buffer was then added to each tube. Additional fluorescence-minus-one (FMO) controls received either no antibody, anti-CD61 only, or anti-CD62P only. Tubes were incubated at room temperature in the dark for 30 minutes. After staining, 0.5 mL of 1% paraformaldehyde in PBS was added to each tube to fix cells. Fixed cells were stored at 2 – 8 °C until analysis within 24 hours of fixation.

2.5 Flow Analysis

Samples were analyzed on a BD FACSCantoII flow cytometer using BD FACSDiva software. Cells were classified as platelets based on their expression of CD61. CD61+ cells were further examined for expression of CD62P+; cells that were both CD61+ and CD62P+ were considered activated platelets. A count of the number of cells in each population was obtained. A total of 10,000 CD61+ cells were analyzed for each sample.

The threshold of fluorescence for the CD61+ and CD62P+ populations were determined by analyzing the FMO controls. As the FMO controls lack either FITC or PerCP-Cy5.5 conjugated antibodies, any fluorescence in those channels in the samples lacking the relevant antibody was deemed background fluorescence. Gates were drawn at the edge of this population, so that cells in the experimental samples more fluorescent than the FMO control were deemed either CD61+ or CD62P+.

2.6 Data Analysis

The number of CD62P+ cells were expressed as a percentage of the CD61+ cells (all platelets) for each sample. Averages and standard deviations of the triplicate tubes of a single sample were obtained. Statistical significance was evaluated using ANOVA and post-hoc tests for multiple comparisons using GraphPad Prism Software (San Diego, CA).

3. RESULTS

3.1 PRP vs Whole Blood

Platelets are highly sensitive to activation by physical perturbation or agitation. Therefore, we first focused on the processes of blood collection and handling to ensure that platelets used for an experiment had minimal levels of activation. We used a positive control, adenosine diphosphate

(ADP), an agonist which activates platelets through G protein-coupled receptor signaling [2], to measure the maximum measurable level of activation of platelets in whole blood or platelet rich plasma. After a short exposure to a titration of ADP concentrations, we carried out antibody staining and flow cytometry to measure the percentage of platelets and activated platelets in each sample (Figure 1). These results showed that PRP samples had a much lower baseline activation level (without ADP) than that of whole blood samples; furthermore, the maximal activation observed in PRP (200 µM ADP) was approximately 20% greater than whole blood. In addition, PRP samples had a greater concentration of platelets, allowing for a clearer identification of CD62P+ than possible in whole blood. These results showed that exposing potential test articles to PRP allowed for a greater dynamic detecting range than exposure to whole blood.

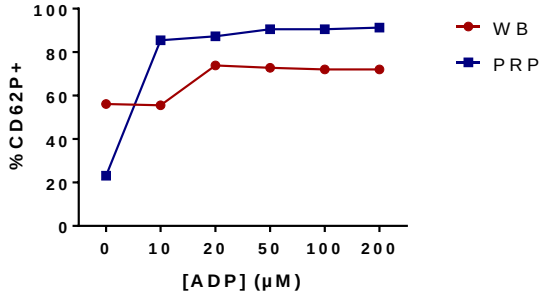

Figure 1. Whole blood (WB) or platelet-rich plasma (PRP) was incubated with the indicated concentrations of adenosine diphosphate (ADP) for 10 minutes at room temperature. The percentage of CD62P-positive platelets was determined over CD61-postive (all platelets).

3.2 Identification of Reference Materials

We next identified reference materials for use as positive and negative controls in the platelet activation assay. We identified several candidates based on their historical usage in other hemocompatibility assays, including black rubber, latex, and high density polyethylene. The materials were prepared by cutting into small pieces suitable to fit into a polypropylene tube. The surface area of the materials was determined and the required volume of PRP necessary to achieve an exposure ratio of 6 cm^2/mL was calculated. Materials were prepared in triplicate and exposed to PRP for 1 hour. The exposed PRP was removed and stained with anti-CD61 / anti-CD62P and analyzed. Five independent experiments were performed and compiled (Figure 2).

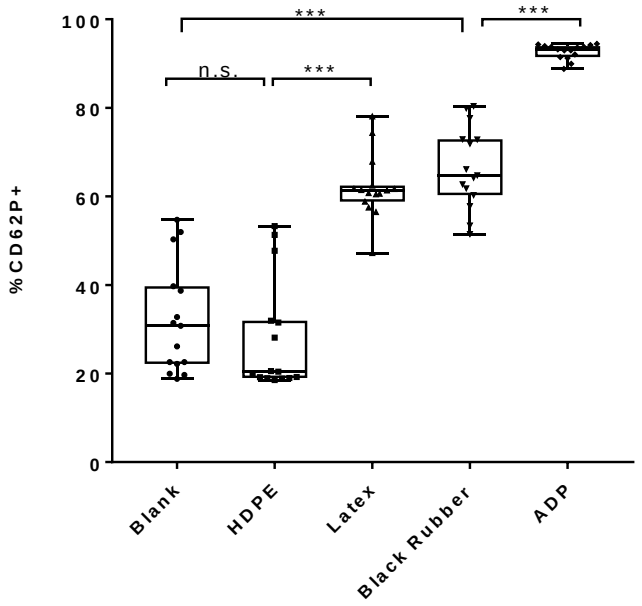

Figure 2. The indicated reference materials were incubated with platelet-rich plasma for one hour. HDPE, Latex, and Black Rubber were incubated at a surface area to volume extraction ratio of 6 cm^2 / mL. PRP alone and 50 µM ADP were used as negative and positive controls, respectively (n.s.: not significant ($p > 0.05$), ***: $p < 0.01$).

These results indicate that both latex and black rubber were suitable as positive reference control materials in the platelet activation assay, with consistent activation levels above baseline. ADP was a robust positive control included in each run to demonstrate that platelets could be competently activated, inducing approximately 90-95% activated platelets. Although none of the materials produced activation as high as the ADP agonist, black rubber had levels of activation significantly greater than the blank and HDPE controls. Furthermore, HDPE consistently had levels of activation similar to that of the plasma control, suggesting its consistency as a negative control material.

3.3 Anti-coagulant Comparison

We next measured the effect of anticoagulant selection on the platelet activation assay. Based on the commonly used anti-coagulants in the standard ISO 10993-4 hemocompatibility assays, we compared the effect of 3.2% sodium citrate with 1 IU/mL heparin (Figure 3). Citrated PRP had a far lower baseline level of activation compared to heparinized PRP. In addition, the maximal level of activation induced by ADP in the citrated PRP was nearly 3-fold that of the heparinized PRP. While the heparinized PRP had similar levels of activation as citrated PRP for many materials, normalization to the baseline revealed that citrated PRP had a much greater separation between positive and negative samples, allowing for characterization of a greater range of potentially intermediate activators found in medical devices.

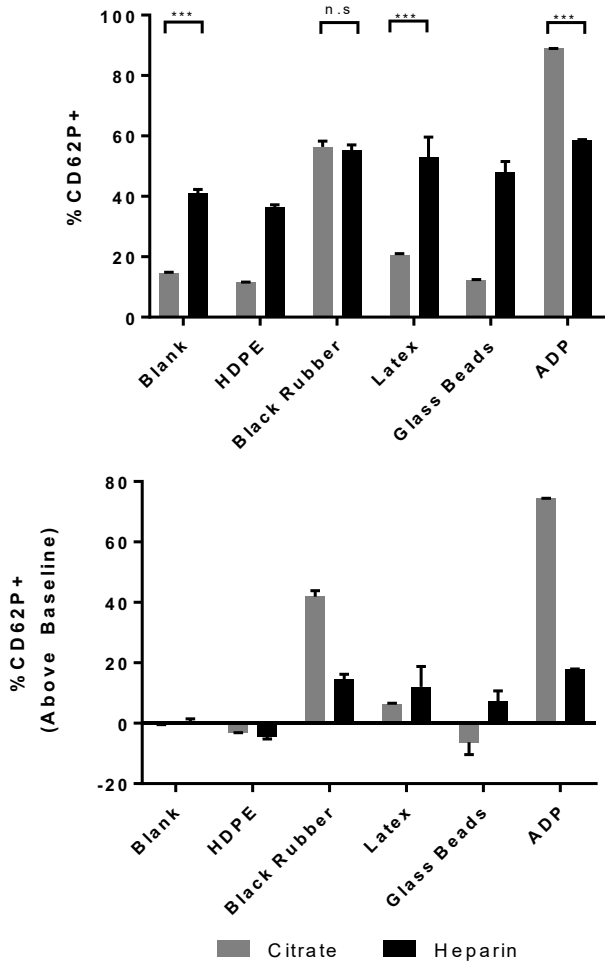

Figure 3. Reference materials were exposed to PRP obtained from whole blood anticoagulated with either 3.2% sodium citrate or 1 IU/mL heparin for 1 hour. PRP alone and 50 μM ADP were used negative and positive controls, respectively. (A): The percentage of CD62P+ platelets in each sample. (B): The amount of platelet activation expressed as a percentage of the activation present in the blank tube. (n.s.: not significant ($p > 0.05$), ***: $p < 0.01$).

3.4 Donor Variability

In general, usage of blood from three donors is required for standard ISO 10993-4 hemocompatibility assays to cover donor variability. Therefore, we performed experiments to compare activation of the reference materials in PRP from three separate donors with the pooled PRP from all three donors (Figure 4). Two-way ANOVA analysis revealed that there was no significant difference between any of the three donors and the pooled PRP, suggesting that pooling donors is an appropriate practice for the platelet activation assay. Moreover, pooling plasma from three independent donors did not cause any increase in the plasma vehicle control owing to inter-donor interactions.

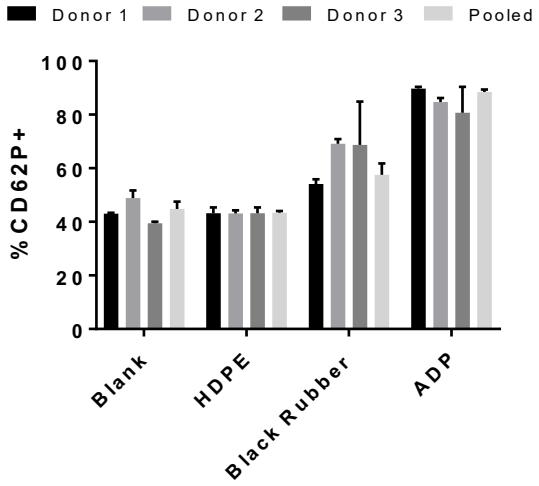

Figure 4. PRP was obtained from three healthy donors and exposed to reference materials and 50 μM ADP for 1 hour. An equal volume of PRP from each donor was combined as a pooled sample and also exposed to the reference materials. The percentage of CD62P-positive platelets was determined by flow cytometry.

4. CONCLUSION

Taken together, we have identified and examined several experimental conditions of a flow cytometry based platelet activation assay. We have identified several reference materials to serve as robust positive and negative controls. The use of platelet rich plasma, in place of whole blood, allows for more precise identification of platelet and activated platelet populations, as well as providing a better dynamic range for assay response. Readily available reference materials were tested and shown to provide robust and consistent levels of activation for use as controls. Furthermore, these reference materials are used in currently established hemocompatibility assays, corroborating the platelet activation assay's place as an integral to the overall assessment of hemocompatibility for medical devices.

Overall, the flow cytometry-based platelet activation assay is an inexpensive and expeditious test to evaluate hemocompatibility for medical devices, allowing for high throughput evaluation using small numbers of devices. As platelet activation occurs as an intermediate step among the standard endpoints of current testing paradigms, the flow cytometry-based assay may provide a more sensitive and nuanced characterization of a device's interactions with blood. Furthermore, our assay is extensible to test for additional markers of platelet activation, or even other biological markers, through use of additional fluorescent antibodies. Further optimization and data collection should be performed to continue evaluation of this assay's predictive capability and correlation with other hemocompatibility tests.

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