Analysis of Platelets by Cytometry  (13-Nov-2004)

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Introduction

Although unimpressive when viewed through the light microscope, platelets are highly complex cells that play a critical role in hemostasis and maintenance of vascular integrity. Clinical disorders of platelet numbers and function are frequently encountered in clinical medicine. Thrombocytopenia results from decreased production, consumption in hemostatic disorders, or immune-mediated destruction. Both hypofunctional and hyperfunctional platelet disorders have been described. Hypofunctional platelets have been associated with congenital defect and clinical diseases. Under a variety of disease conditions platelets can become activated in vivo and can initiate thrombotic events detrimental to the well being of the animal. To advance the clinical evaluation of platelet disorders, 3 major types of tests are needed. First, tests are needed to differentiate thrombocytopenia associated with destruction or consumption from that associated with decreased production. Secondly, tests for detection of anti-platelet antibodies would greatly facilitate the diagnosis of primary and secondary immune-mediated thrombocytopenia. Thirdly, tests to detect platelets that are primed or activated in vivo are important for detection of prothrombotic states. Techniques for assessment of each of these categories will be discussed.

Sample Collection

Blood is aspirated into sterile plastic syringes and is immediately transferred to evacuated tubes containing EDTA. Prostaglandin E, (5 uM) has been added to prevent ex vivo platelet activation but has been reported to reverse binding of fibrinogen to activated platelets [1,2]. Blood should be stored at room temperature and processed within 4 - 12 hours [2,3]. Either platelet-rich plasma or whole blood can be used. If whole blood is used, 50 ul of blood is placed in a sterile plastic 6 ml test tube and 1.0 ml of erythrocyte lysis buffer, containing 0.5 % paraformaldehyde is added. As a positive control, platelets in whole blood or platelet-rich plasma can be activated, prior to fixation, by addition of phorbol myristate acetate (10 - 20 ng/ml) and incubated for 20 minutes at 37°C. After fixation, platelets are washed by addition of at least a 200 fold excess of physiologic buffered saline solution consisting of Dulbecco's phosphate buffered saline, 1% normal goat serum, 5 mM sodium azide, and 0.1% glycine (FACS buffer). The FACS buffer must be filtered through a 0.45 um pore syringe filter just before use to remove particulate debris [3]. After centrifugation at 435 x g at 8°C for 15 minutes, platelets are resuspended in 100 ul of FACS buffer.

Activated Platelet Technique

Platelet shape change, aggregates, microparticles - Platelets are analyzed by use of a flow cytometer [3]. The platelet population is displayed as log forward-angle vs. log side-angle light scatter plots (Fig 1). Using unactivated platelets, a gate is set to include greater than 95% of the platelets. Two additional gates are set to detect aggregated platelets and platelet microparticles [2,3].
Samples spiked with low numbers of erythrocytes should be used to avoid inclusion of these cells in the platelet aggregate gate. The percentage of cells in each gate is recorded. Platelet shape change occurs when platelets are activated. The normal platelet cluster is oval in shape, whereas, the activated platelet cluster is flattened and elongated (Fig 1).

**P-selectin** - P-selectin is normally present on the internal surface of alpha granules and is transported to the cell surface when platelets degranulate. Most species have very little P-selectin expressed on the cell membrane of unactivated platelets. However, the horse has been reported to express significant amounts of P-selectin on unactivated platelets [4]. These assays were done on platelets fixed in 4% paraformaldehyde. Because paraformaldehyde activates horse platelets, further studies, using unfixed platelets, are needed to evaluate equine P-selectin expression. To detect platelet surface-associated P-selectin, platelet suspensions are incubated with 100 ul of a 1:100 dilution of affinity purified mouse anti-canine P-selectin (MD6, IgG-1, 0.45 mg/ml, (provided by Dr. C. Wayne Smith, Baylor College of Medicine, Houston, TX) or an isotype-matched negative control antibody for 30 minutes [3]. Platelets are washed by addition of at least a 200 fold excess of FACS buffer. After centrifugation at 435 x g at 8°C for 15 minutes, platelets are resuspended in 100 ul of FACS buffer. Thereafter, 100 ul of a 1:150 dilution of fluorescein-labeled goat anti-mouse IgG (clone STAT70, Serotec USA, Washington, DC) is added and platelets are incubated in the dark at room temperature for 20 minutes. After incubation, 400 ul of FACS buffer is added and tubes are stored in the dark at 4°C and analyzed within 24 hour.

Platelets are analyzed by use of a flow cytometer [3]. A histogram plot with log green fluorescence intensity on the x-axis and cell number on the y-axis is used to define median fluorescence intensity of the main platelet population and the percentage of platelets with increased fluorescence intensity. Using negative control platelets, a gate is set to detect cells with increased fluorescence intensity so that less than 5% of unactivated platelets are included in the gate. Platelet aggregates and platelet microparticles are not included in the determination of platelet fluorescence intensity.

Platelets collected in EDTA had minimal expression of P-selectin, whereas, platelets collected in sodium citrate had a greater median fluorescence intensity [3]. Fixation with 0.5% paraformaldehyde before labeling platelets with anti-P-selectin did not affect antibody binding or the percentage of platelet aggregates and microparticles. Storage of platelet-rich plasma at 4°C for 4 hours did not affect antibody binding or the percentage of platelet aggregates or microparticles. However, after 24 hours of storage, the median fluorescence intensity of both unactivated and activated platelets tended to increase. Addition of 10, 20, or 50 ng/ml PMA resulted in a progressive increase in the median fluorescence intensity of the platelet population and a modest but progressive increase in the percentage of fluorescent platelets. Addition of 10 ng/ml of PMA resulted in only a slight increase in platelet aggregates, whereas, addition of 20 and 50 ng/ml of PMA resulted in a greater increase in the number of platelet aggregates (Fig 1).

Values obtained from flow cytometers vary considerably based on the instrument setting and day-to-day variation. Therefore universal reference intervals cannot be established. It is recommended that one or more normal control animals be included in each run. In previous studies of healthy dogs, the median fluorescence intensity and percent platelets with increased fluorescence intensity were only slightly greater than that of negative control platelets [3]. This indicates that P-selectin is not expressed on unactivated platelets and that routine methods of collection and processing of blood samples do not activate canine platelets. When platelets were activated by addition of 10 ng/ml PMA, a 10-fold increase in median fluorescent intensity was observed (Table 1) Incubation with PMA increased the number of platelet aggregates and microparticles only slightly.
Annexin V - When platelets become activated, phosphatidylserine moves from the inner leaflet of the cell membrane bilayer to the outer leaflet [1]. Increased expression of phosphatidylserine on the outer leaflet can be detected by binding of fluorescein-labeled annexin V. In horses, formalin fixation of platelets has been shown to increase annexin V binding [1]. The technique is relatively simple. Twenty microliters of platelet-rich plasma is combined with 80 ul of physiologic buffered saline, 250 ul of annexin V binding buffer, and 5 ul fluorescein-labeled annexin V. After incubation for 15 minutes at room temperature in the dark, the reaction is stopped by addition of 750 ul of HEPES buffer. Samples are analyzed as described for P-selectin.

MPC and PCDW - Recently developed tests for automated flow cytometric detection of activated platelets include the mean platelet component concentration (MPC) and the mean platelet component distribution width (PCDW) [5,6]. The MPC technique is dependent on determining platelet refractive index which is linearly related to platelet density. Activated platelets degranulate reducing platelet density and, therefore, lowering the MPC. The PCDW technique measures variation in platelet density. The presence of both normal and degranulated platelets in the blood leads to variation in granularity which results in a high PCDW. The advantage of these tests is that they can be routinely determined by the ADVIA 120 hematology analyzer. Samples are collected using EDTA as the anticoagulant and analyzed within 4 hours. Species specific software, that is provided by the manufacturer, should be used.

Relationship between P-selectin, MPC, and MCDW - To determine the extent to which activated platelets, detected by determination of P-selectin, correlated with automated platelet measurements of activated platelets, we evaluated 20 dogs with inflammatory diseases [7]. When the MPC was compared to P-selectin mean fluorescence intensity (MFI), the correlation coefficient was 0.62 (Fig 1c; P < 0.001). The correlation coefficient between the percentage of platelets with increased MFI and MPC was 0.52. When individual dogs were compared, all dogs that were P-selectin positive also had an abnormal MPC, however, 4 dogs, that had normal P-selectin MFI had abnormal MPC results. The correlation between PCDW and P-selectin MFI (r = 0.30) was low. These data indicate that MPC gives a positive result for activated platelets for more dogs than does the P-selectin test. One possible explanation is that activated, platelets shed surface P-selectin but continue to circulate as poorly granular platelets.

Platelet-leukocyte aggregates - Activated platelet bind to neutrophils and monocytes primarily through the interaction of P-selectin on the platelet surface with PSGL-1 on the leukocyte surface [8]. Platelet-neutrophil aggregates are identified by labeling platelets with phycoerythrin and leukocytes with fluorescein [9]. Co-labeling of leukocytes with both stains detects leukocytes with adherent platelets. One hundred microliters of blood is placed in a plastic tube, 2 ml of an erythrocyte lysis buffer containing 0.5 % paraformaldehyde is added, and samples are incubated at room temperature for 10 minutes (do not centrifuge before fixation). Thereafter, samples are centrifuged for 3 minutes at 1200 x g, washed twice in FACS buffer, and resuspended in 100 ul of FACS buffer. Saturating concentrations (10 ul) of mouse anti-horse GPIIbIIIa is added and samples are incubated at 22 C for 30 minutes. After washing, saturating concentrations of a phycoerythrin-labeled sheep anti-mouse IgG is added and incubated in the dark for 30 minutes at 22 C. Five percent mouse serum is added for 10 minutes to block nonspecific binding. After washing, fluorescein-labeled anti CD11/CD18 is added and incubated for 30 minutes. Negative control samples are incubated with a isotype-matched mouse antibody. Neutrophils are identified by use of forward and side angle light scatter. A gate is set to include the neutrophil and monocyte population. A scatter plot with log green fluorescence intensity on one axis and red fluorescence intensity on the other axis is constructed. The percentage of leukocytes labeling with both stains is recorded.

Other techniques - Other flow cytometric tests for activated platelets that have been described include detection of fibrinogen binding to cat and horse platelets using anti-human fibrinogen [10,11], and detection of a receptor-induced binding site on fibrinogen in dogs [12]. During platelet activation, glycoprotein IIbIIIa undergoes an activation-induced conformational change that permits binding to fibrinogen. A monoclonal antibody that recognizes a receptor-induced binding site on canine fibrinogen has been described [12]. Activated horse platelets have been reported to release thrombospordin [13].
Flow Cytometric Detection of Immature Platelets
As with immature erythrocytes, immature platelets are large and have retained ribonucleic acid (RNA) content. Measures of immature platelets include reticulated platelets, mean platelet volume (MPV), and platelet distribution width (PDW). Mean platelet volume is a measure of platelet size and PDW is a measure of variation in platelet size. Rapid mobilization of platelets from the bone marrow, associated with platelet consumption or destruction, results in increased MPV and PDW values. Mean platelet volume and PDW are measured as part of an automated complete blood counts [5,14]. In a clinical study of 60 thrombocytopenic dogs, the sensitivity of MPV for detection of increased thrombopoiesis was reported to be 88% and the specificity was 80% [15]. The predictive value of a positive test was 96% and the predictive value of a negative test was 57%.

Reticulated platelets have been detected by staining retained RNA with various dyes and analyzing by use of a flow cytometer. The most frequently used dye is thiaazole orange [2,16,17]. Blood samples are collected in EDTA. Whole blood is lysed and fixed by incubation of blood with an erythrocyte lysis buffer containing 0.5% paraformaldehyde for 10 minutes. After washing, platelets should be resuspended at approximately 1 x 10⁶/ml. Platelets can be stored for several days at 4°C before analysis. Approximately 1 hour before analysis, 500 ul of thiazole orange is added. The platelet population is identified in log forward-angle vs. log side-angle light scatter plots. A scatter plot or histogram plot is used to display the fluorescence intensity of the platelet population. Using a negative control sample incubated without addition of thiazole orange, a gate is set to detect platelets with increased fluorescence intensity. Increased thrombopoiesis is frequently associated with an increase in the percentage of platelets with increased fluorescence intensity. Alternatively, mean fluorescence intensity of the entire platelet population and total reticulated platelets/ul are frequently not increased. Reported values for normal dogs varied between 3.9 and 15% and for horses varied between 0.9 and 3.4% [16,17].

Platelet surface-associated Immunoglobulin G (PSAIgG)
PSAIgG has been used for detection of primary and secondary immune-mediated thrombocytopenia (IMP) [2]. In this technique, washed platelets are incubated with FITC-conjugated anti-canine IgG. Background fluorescence is evaluated by labeling platelets with an isotype-matched FITC-labeled mouse IgG. Because of the relatively high background fluorescence, PSAIgG values should be corrected by subtracting background fluorescence. The mean fluorescence intensity of the main platelet population (excluding platelet aggregates and microparticles) and the percentage of platelets with increased fluorescence intensity is recorded.

Summary
Despite the paucity of studies of activated platelets in animals, P-selectin, platelet-leukocyte aggregates, MPC, MPV, reticulated platelets, and PSAIgG appear to be appear to provide useful clinical data for evaluation of platelet disorders. All techniques can be determined on EDTA-anticoagulated samples with no special handling procedures. The MPC and MPV have the distinct advantage of being included as a part of an automated complete blood count. However, one potential disadvantage of the MPC is its sensitivity. Eighty percent of dog with septic and nonseptic inflammatory diseases had low MPC abnormal values and abnormal values did not appear to predict the severity of disease [7]. Hopefully, larger studies and clinical experience will permit us to define values for MPC and P-selectin that predict prothrombotic conditions.
References


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