# Evaluation of flow cytometric and automated methods for detection of activated platelets in dogs with inflammatory disease

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**Objective**—To evaluate platelet surface-associated P-selectin, mean platelet component concentration (MPC), mean platelet component distribution width (MPCDW), mean platelet volume (MPV), and platelet distribution width (PDW) for detection of activated platelets in dogs with septic and nonseptic inflammatory disease.

**Animals**—20 healthy dogs and 20 dogs with septic and nonseptic inflammatory disease.

**Procedures**—Platelet surface-associated P-selectin (expressed as the median fluorescence intensity [MFI] of the platelet population), MPC, MPCDW, MPV, and PDW were determined in 20 healthy adult dogs, and reference ranges were calculated. These parameters were also determined in 11 dogs with nonseptic and 9 dogs with septic inflammatory disease and evaluated to determine which parameters were useful for detection of activated platelets.

**Results**—12 dogs with inflammatory disease had P-selectin greater than the upper limit of the reference range, whereas 16 dogs with inflammatory disease had MPC lower than the lower limit of the reference range. All dogs in which P-selectin was greater than the upper limit of the reference range had MPC lower than the lower limit of the reference range. The correlation coefficient for P-selectin and MPC was 0.62. Differences in the MPCDW, MPV, and PDW in most dogs with inflammatory disease (compared with healthy dogs) were found; however, the correlation coefficients for P-selectin and MPCDW, MPV, and PDW were low.

**Conclusions and Clinical Relevance**—Platelet surface-associated P-selectin and MPC appeared to be useful to detect activated platelets in most dogs with septic and nonseptic inflammatory disease. (*Am J Vet Res* 2005;66:325–329)

Platelet activation in vivo may play an important role in the pathogenesis of a variety of conditions in dogs. Platelet aggregometry has been used to detect hyperaggregable platelets in dogs with multicentric lymphosarcoma, other malignancies, nephrotic syndrome, and heartworm disease.[1–4] Hypoaggregable platelets have been detected in dogs with liver disease and experimentally induced pancreatitis.[5,6] No changes in platelet aggregation were detected in dogs with uremia.[7]

Flow cytometry has been used to detect activation-dependent markers on circulating platelets.[8–12] Activation markers detected on the surface of canine platelets include P-selectin and fibrinogen bound to glycoprotein IIb-IIIa.[11–13] P-selectin is a component of the $\alpha$-granule membrane and is expressed on the platelet surface after $\alpha$-granules are secreted.[9,14] Two anti-canine P-selectin antibodies and 2 antibodies that cross-react with canine platelet P-selectin have been described.[11,15,16]

We have described a flow-cytometric technique for detection of activated platelets in healthy dogs.[8] Activated platelets were detected via incubation of platelets with a mouse anti-canine P-selectin antibody followed by incubation with a fluorescein-labeled, goat anti-mouse IgG antibody. The **median fluorescence intensity (MFI)** of the platelet population and the percentage of platelets with increased fluorescence intensity increased when platelets were activated via addition of **phorbol myristate acetate (PMA)**.

Recently developed techniques for automated detection of activated platelets include determinations of **mean platelet component concentration (MPC)** and **mean platelet component distribution width (MPCDW)**.[17,18] The technique for determining MPC measures the refractive index of platelets; refractive index is linearly related to platelet density. When activated platelets degranulate, platelet density decreases and therefore the MPC decreases. The MPCDW is an indicator of the variation in platelet density; therefore, if both nondegranulated and degranulated platelets are present in the circulation, the MPCDW is high. **Mean platelet volume (MPV)** is a measure of platelet size, and **platelet distribution width (PDW)** is an indicator of the variation in platelet size. The advantage of use of these variables is that they can be routinely determined by use of an automated hematology analyzer[a]; however, the usefulness of these variables for detection of activated platelets in dogs has not been extensively evaluated.

The purpose of the study reported here was to evaluate platelet surface-associated P-selectin, MPC, MPCDW, MPV, and PDW for detection of activated platelets in dogs with septic and nonseptic inflammatory disease.

## Materials and Methods

**Blood samples**—Blood samples submitted to the clinical laboratory at the University of Minnesota Veterinary

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Clinical Center were used. Blood samples were obtained from 20 healthy adult dogs that had not been treated with any drugs for at least 10 days prior to sampling. Dogs were considered healthy on the basis of no abnormal findings on physical examination, neutrophil and platelet counts within the respective reference ranges for the laboratory, a band neutrophil count < 200 cells/µL, and absence of morphologic abnormalities on examination of blood smears.

Blood samples were also collected from 20 dogs with inflammatory disease. The criteria for selection of dogs with inflammatory disease included the presence of fever, a neutrophil count greater than the upper limit of the reference range for the laboratory, and a band neutrophil count > 1,000 cells/µL. For 20 dogs with inflammatory disease, bacterial infections were identified in 9 (via cytologic examination or bacterial culture of appropriate samples); 11 dogs had inflammatory disease that was believed to be unassociated with bacterial infection.

**Platelet preparation**—Blood samples for all determinations were collected into sterile plastic syringes and immediately transferred to evacuated tubes containing EDTA. Within 30 minutes of collection, blood was centrifuged at 300 × *g* at 20°C for 10 minutes and platelet-rich plasma was removed by use of a sterile plastic Pasteur pipette.[8] Fifty microliters of platelet-rich plasma was placed in sterile plastic 6-mL polypropylene test tubes. Platelets were washed via addition of at least a 200-fold excess of a buffer solution consisting of Dulbecco phosphate-buffered saline solution, 1% normal goat serum, 5 mM sodium azide, and 0.1% glycine. Just prior to use, the buffer solution was filtered through a 0.45-µm-pore syringe filter to remove small particulate material. After centrifugation at 435 × *g* at 8°C for 15 minutes, platelets were resuspended in 100 µL of buffer solution.

**Detection of platelet surface-associated P-selectin**—Platelets were incubated with 100 µL of a 1:100 dilution of affinity-purified, monoclonal, mouse anti-canine P-selectin antibody[b] or with an isotype-matched antibody (negative control)[c] for 30 minutes.[8] Platelets were washed once via addition of at least a 200-fold excess of buffer solution. After centrifugation at 435 × g at 8°C for 15 minutes, platelets were resuspended in 100 µL of buffer solution. One hundred microliters of a 1:150 dilution of fluorescein-labeled, goat anti-mouse IgG antibody[d] was added, and platelets were incubated in the dark at 21°C for 20 minutes. After incubation, 400 µL of buffer solution was added, tubes were stored in the dark at 4°C, and analyses were performed within 1 hour.

Platelets were evaluated by use of a flow cytometer.[e] Platelet populations were displayed on log forward-angle versus log side-angle light scatter plots as described.[8] A gate was set so that > 95% of the platelet population was included. A histogram with log values for green fluorescence intensity on the x-axis and platelet number on the y-axis was constructed. By use of platelets from a healthy dog, a gate was set to detect platelets with increased fluorescence intensity. The percentage of platelets with increased fluorescence intensity and the MFI of the platelet population were recorded. For use as positive controls, platelets from all dogs were activated via incubation with PMA (final concentration, 10 ng/mL) at 37°C for 10 minutes. Phorbol myristate acetate was chosen as the agonist because of its ability to induce slow progressive activation and degranulation of platelets without inducing aggregation.[8]

**Platelet count, MPC, MPCDW, MPV, and PDW**—The platelet count, MPC, MPCDW, MPV, and PDW were determined by use of an automated hematology analyzer[a] equipped with multispecies software. The methods used were those recommended by the manufacturer.[17,18]

**Data and statistical analyses**—Median and 95% **confidence interval (CI)** values were calculated for each variable in each group of dogs. For the purposes of our study, data for the 20 healthy dogs were used to calculate reference ranges for each variable. Outliers, defined as values that exceeded one third of the range ± 2 SD, were not identified. Most of the data were not normally distributed; therefore, the reference ranges for all data, including the normally distributed data, were calculated and reported as the 95% CI.

Correlation coefficients were calculated to assess the relationship between platelet surface-associated P-selectin and MPC, MPCDW, MPV, and PDW. Values of variables in dogs with inflammatory disease were compared with values of corresponding variables in healthy dogs by use of the Wilcoxon matched pair test.[f] Values of $P < 0.05$ were considered significant.

## Results

**Reference ranges for platelet variables**—Reference ranges were calculated for platelet sur-

Table 1—Median (95% confidence interval) values for platelet parameters in 20 adult dogs with inflammatory disease and 20 healthy adult dogs.

| Parameter | Phorbol myristate acetate | Nonseptic inflammation (n = 11) | Septic inflammation (9) | Healthy (20) |
|---|---|---|---|---|
| Platelet count (× 10³/µL) | – | 214 (128–285) | 189 (86–299) | 263 (204–398) |
| P-selectin (MFI) | – | 11.0 (2.3–16.4)* | 7.7 (2.5–34.2) | 1.9 (0.9–3.0) |
| | + | 21.6 (5.0–38.3) | 26.9 (4.1–34.6) | 19.7 (7.0–59.2) |
| Platelets with increased fluorescence intensity (%) | – | 26.8 (9–42)* | 21 (3–53)* | 5.8 (0.4–14.6) |
| | + | 42 (8–59) | 37 (5–62) | 23.3 (8–23) |
| MPC (g/dL) | – | 18.3 (15.2–20.8)* | 21.1 (19.9–23.2) | 21.9 (20.9–22.5) |
| MPCDW (g/dL) | – | 6.09 (4.22–7.6) | 5.58 (3.8–7.7) | 4.31 (4.23–4.72) |
| MPV (fL) | – | 17.7 (9.4–28.9)* | 17.8 (13.3–29.3)* | 10.1 (8.4–11.5) |
| PDW (%) | – | 67.3 (62.2–72.0)* | 70.4 (65.3–76.6)* | 55.8 (54.4–60.0) |

*Significantly ($P < 0.05$) different from value in healthy dogs.
– = Not stimulated with phorbol myristate acetate. + = Stimulated with phorbol myristate acetate. MFI = Median fluorescence intensity of the platelet population. MPC = Mean platelet component concentration. PCDW = Mean platelet component distribution width. MPV = Mean platelet volume. PDW = Platelet distribution width.



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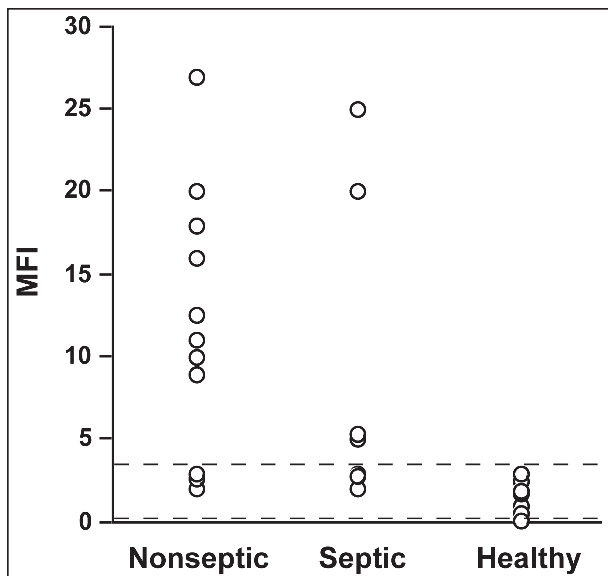


Figure 1—Datum points for median fluorescence intensity (MFI) of the platelet population (platelet surface-associated P-selectin) in healthy dogs (n = 20) and dogs with nonseptic (11) and septic (9) inflammatory disease. Dashed lines indicate the upper and lower limits of the reference range calculated by use of values in the 20 healthy dogs. Some datum points overlap.

face-associated P-selectin (expressed as MFI of the platelet population and as the percentage of platelets with increased fluorescence intensity), MPC, MPCDW, MPV, and PDW by use of data from the 20 healthy dogs (**Table 1**). Median fluorescence intensity of the platelet population was low in all healthy dogs. When platelets were activated via incubation with PMA, a 3-fold or greater increase in P-selectin of the platelet population was observed in all healthy dogs.

**Evaluation of platelet variables in dogs with inflammatory disease**—The platelet count in 6 dogs with inflammatory disease was lower than the lower limit of the reference range. P-selectin of the platelet population was greater than the upper limit of the reference range in 8 dogs with nonseptic inflammatory disease and 4 dogs with septic inflammatory disease (Table 1; **Figure 1**). The percentage of platelets with increased P-selectin was greater than the upper limit of the reference range in 8 dogs with nonseptic inflammatory disease and 5 dogs with septic inflammatory disease. Activation of platelets by use of PMA resulted in an increase in P-selectin from baseline values in 8 dogs with nonseptic inflammatory disease and 6 dogs with septic inflammatory disease. Mean platelet component concentration was lower than the lower limit of the reference range in 11 dogs with nonseptic inflammatory disease and 5 dogs with septic inflammatory disease (Table 1; **Figure 2**). Platelet component distribution width was greater than the upper limit of the reference range in 9 dogs with nonseptic inflammatory disease and 5 dogs with septic inflammatory disease. Mean platelet volume was greater than the upper limit of the reference range in 9 dogs with nonseptic inflammatory disease and 9 dogs with septic inflammatory disease. The PDW was greater than the upper limit of the reference range in all 20 dogs with inflammatory disease.

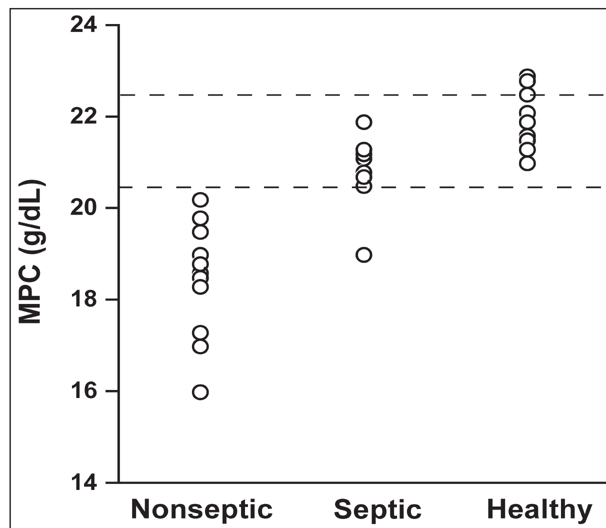


Figure 2—Datum points for mean platelet component concentration (MPC) in healthy dogs and dogs with nonseptic and septic inflammatory disease. Dashed lines indicate the upper and lower limits of the reference range calculated by use of values in the 20 healthy dogs. Some datum points overlap.

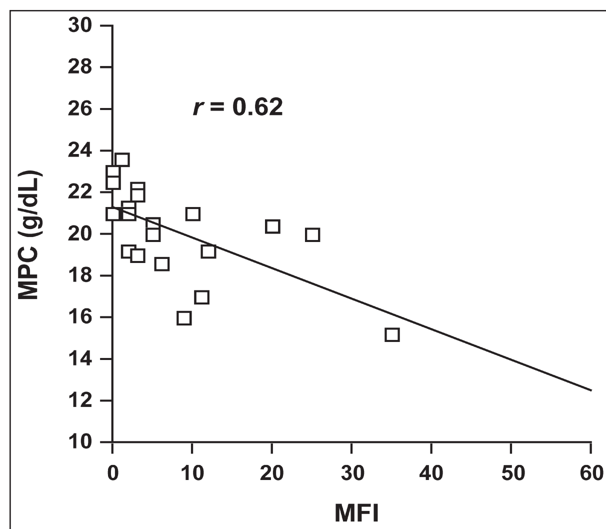


Figure 3—Correlation of MFI of the platelet population and MPC in 20 dogs with inflammatory disease.

**Correlation between parameters**—To determine the extent to which activated platelets detected via determination of platelet surface-associated P-selectin correlated with the other platelet variables, we calculated correlation coefficients by use of data from dogs with inflammatory disease. The correlation coefficient for MPC and P-selectinof the platelet population was 0.62 (**Figure 3**; $P < 0.001$). The correlation coefficient for the percentage of platelets with increased P-selectin and MPC was 0.52. When individual dogs were considered, all dogs that had P-selectin greater than the upper limit of the reference range also had MPC lower than the lower limit of the reference range; however, 4 dogs that had P-selectin within the reference range had MPC lower than the lower limit of the



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reference range. The correlation coefficients for P-selectin and MPCDW ($r = 0.30$), P-selectin and MPV ($r = 0.20$), and P-selectin and PDW ($r = 0.16$) were low.

## Discussion

Detection of activated platelets may be a sensitive method for early diagnosis of prothrombotic conditions. Platelet surface-associated P-selectin has been used as a platelet activation marker in several species.[8,10,11] This method appears to be clinically applicable but requires a flow cytometer and approximately 60 minutes of sample preparation time.[8] The MPC and MPCDW have the advantage of being available as parts of a routine CBC.[17] As a result, hematologic data can be generated quickly and inexpensively.

Four dogs with inflammatory disease had MPC lower than the lower limit of the reference range; however, these dogs had P-selectin within the reference range. This resulted in a lower correlation for the 2 variables in dogs ($r = 0.62$), compared with that for humans ($r = 0.85$).[19] These results suggest that MPC may be more sensitive than P-selectin for detection of platelet activation or that MPC results may be false positive. On the basis of what is known about these variables, the MPC would be expected to be a more sensitive test. In a study[20] in which baboon platelets were activated ex vivo via addition of thrombin and then reinfused into the same baboons, reinfused platelets rapidly lost their surface-associated P-selectin but continued to circulate and function. These degranulated platelets would be detected via determination of the MPC but not via determination of the P-selectin; therefore, loss of P-selectin from the surface of activated platelets could explain the greater sensitivity of MPC.

When platelets were activated with low concentrations of PMA, platelets from all healthy dogs had a 3-fold or greater increase in P-selectin of the platelet population. However, platelets of 6 dogs with inflammatory disease did not respond to PMA. These results suggest that some dogs with inflammatory disease may have circulating platelets that are hypofunctional. These circulating platelets may have been previously partially activated, leaving them refractory to further activation.

Regardless of differences between P-selectin of the platelet population and MPC, our results indicate that at least 60% of dogs with inflammatory disease had circulating activated platelets. This percentage is higher than that reported for humans with inflammatory disease.[21–23] In 1 study,[21] 43% of humans with Crohn disease or inflammatory bowel disease had activated platelets. In other studies,[22,23] most humans with uncomplicated sepsis did not have activated platelets; however, most patients with severe sepsis associated with multiple organ failure had circulating activated platelets, suggesting that platelet activation and lesion severity were positively associated.[22,23]

Values for MPCDW, MPV, and PDW were outside of the respective reference ranges in most dogs with inflammatory disease. The MPCDW that was greater than the upper limit of the reference range was likely the result of a subpopulation of degranulated platelets in circulation. The MPV and PDW were greater than the upper limit of their respective reference ranges, which was likely the result of release of large platelets from bone marrow in response to greater demand for platelets.[24] The poor correlation of these variables with P-selectin indicates that they are not reliable predictors of the activation status of platelets. However, our results indicate that determination of both platelet surface-associated P-selectin (MFI) and MPC may be useful for detection of activated platelets in dogs.

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a. Advia 120, Bayer Diagnostics Division, Tarrytown, NY.
b. Clone MD6, IgG1, generously provided by Dr. C. Wayne Smith, Baylor College of Medicine, Houston, Tex.
c. MCA928, Serotec Inc, Raleigh, NC.
d. Clone STAT70, Serotec USA, Washington, DC.
e. FACS Calibur, Becton Dickenson Co, Pleasanton, Calif.
f. Stat View 4.01, Abacus Concepts Inc, Berkeley, Calif.

## References


1. Thomas JS, Rogers KS. Platelet aggregation and adenosine triphosphate secretion in dogs with untreated multicentric lymphoma. *J Vet Intern Med* 1999;13:319–322.
2. McNiel EA, Ogilvie GK, Fettman MJ, et al. Platelet hyperfunction in dogs with malignancies. *J Vet Intern Med* 1997;11:178–182.
3. Boudreaux MK, Dillon AR, Spano JS. Enhanced platelet reactivity in heartworm-infected dogs. *Am J Vet Res* 1989;50:1544–1547.
4. Green RA, Russo EA, Greene RT, et al. Hypoalbuminemia-related platelet hypersensitivity in two dogs with nephrotic syndrome. *J Am Vet Med Assoc* 1985;186:485–488.
5. Willis SE, Jackson ML, Meric SM, et al. Whole blood platelet aggregation in dogs with liver disease. *Am J Vet Res* 1989;50:1893–1897.
6. Jacobs RM, Murtaugh RJ, Fertel RH. Platelet function in experimentally induced pancreatitis in the dog. *Thromb Haemost* 1986;55:197–200.
7. Forsythe LT, Jackson ML, Meric SM. Whole blood platelet aggregation in uremic dogs. *Am J Vet Res* 1989;50:1754–1757.
8. Moritz A, Walcheck BK, Weiss DJ. Flow cytometric detection of activated platelets in the dog. *Vet Clin Pathol* 2003;32:6–12.
9. Mickelson AD. Flow cytometry: a clinical test of platelet function. *Blood* 1996;87:4925–4936.
10. Ruf A, Patscheke H. Flow cytometric detection of activated platelets: comparison of determining shape change, fibrinogen binding, and P-selectin expression. *Semin Thromb Hemost* 1995;21:146–151.
11. Yeo EL, Gemmel CH, Sutherland DR, et al. Characterization of canine platelet P-selectin (CD62) and its utility in flow cytometry platelet studies. *Comp Biochem Physiol* 1993;105B:625–636.
12. Boudreaux MK, Panangala VS, Bourne C. A platelet activation-specific monoclonal antibody that recognizes a receptor-induced binding site on canine fibrinogen. *Vet Pathol* 1996; 33:419–427.
13. Weiss DJ, Mirsky ML, Evanson OA, et al. Platelet kinetics in dogs treated with a glycoprotein IIb/IIIa peptide antagonist. *Toxicol Pathol* 2000;28:310–316.
14. Hamburger SA, McEvers RP. GMP-140 mediates adhesion of stimulated platelets to neutrophils. *Blood* 1990;75:550–558.
15. Dore M, Hawkins HK, Entman ML, et al. Production of a monoclonal antibody against canine GMP-140 (P-selectin) and studies of its vascular distribution in canine tissues. *Vet Pathol* 1993;30:213–222.
16. Dore M, Korthuis RJ, Entman ML, et al. P-selectin mediates spontaneous leukocyte rolling in vivo. *Blood* 1993;82:1308–1316.
17. Macey MG, Carty E, Webb L, et al. Use of mean platelet component to measure platelet activation in the ADVIA 120 hematology system. *Cytometry* 1999;38:250–255.
18. Zelmanovic D, Hetherington EJ. Automated analysis of feline platelets in whole blood including platelet count, mean platelet volume and activation state. *Vet Clin Pathol* 1998;27:2–9.
19. Chapman ES, Sorrette M, Hetherington E, et al. A rapid




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automated flow cytometric method to measure activated degranulated platelets by density determination. *J Thromb Haemost* 2003; 89:1004–1015.

20. Mickelson AD, Barnard MR, Hechtman HB, et al. In vivo tracking of platelets: circulating degranulated platelets rapidly lose surface P-selectin but continue to circulate and function. *Proc Natl Acad Sci* 1996;93:11877–11882.

21. Collins CE, Cahill MR, Newland AC, et al. Platelets circulate in an activated state in inflammatory bowel disease. *Gastroenterology* 1994;106:840–845.

22. Gawaz M, Fateh-Moghadam S, Pilz G, et al. Severity of multiple organ failure (MOF) but not sepsis correlates with irreversible platelet degranulation. *Infection* 1995;23:16–23.

23. Gawaz M, Fateh-Moghadam S, Pilz G, et al. Platelet activation and interaction with leukocytes in patients with sepsis or multiple organ failure. *Eur J Clin Invest* 1995;25:843–851.

24. Sullivan PS, Manning KL, McDonald TP. Association of mean platelet volume and bone marrow megakaryocytopoiesis in thrombocytopenic dogs: 60 cases (1984–1993). *J Am Vet Med Assoc* 1995;206:332–334.



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