Detection of activated platelets in canine blood by use of flow cytometry

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Thromboembolism is associated with a high risk of death in affected patients and is thought to develop in conditions causing blood stasis, vascular injury, or hypercoagulation. In dogs, thromboembolism develops in several diseases, including immune-mediated hemolytic anemia, protein-losing enteropathy, and nephrotic syndrome, and other inflammatory events. Detection of thromboembolism cannot be obtained by use of 1 clinical test but is determined on the basis of a combination of clinical signs, physical examination findings, and diagnostic test results. A diagnosis is often made after a thromboembolic episode has occurred.

Coagulation is propagated and amplified on the platelet surface with expression of platelet phosphatidylserine and exposure of platelet receptors for factors VIIIa and Va. Phosphatidylserine is a phospholipid with a negative charge that can be found primarily on the inner platelet membrane during a resting state. With platelet activation, an ATP-dependent translocase transports phosphatidylserine to the outer surface. The exposure of phosphatidylserine creates a negatively charged surface propagating coagulation by promoting binding of FVIIIa and FXa to the platelet surface. Phosphatidylserine binds FXa and FVa. The assembly of phosphatidylserine, FVIIIa, and FIXa forms the tenase complex, which activates FX. Then FXa forms the prothrombinase complex with FVa and phosphatidylserine generating thrombin, which causes a positive feedback loop that activates FVIII, FV, and platelets, leading to further activation and coagulation.

Platelets are also involved with inflammation and contain several inflammatory mediators, including P-selectin. P-selectin is stored in platelet α-granules as a granule membrane protein and also in Weibel-Palade bodies of endothelial cells. P-selectin is secreted to the platelet surface during activation where it can bind a ligand, PSGL-1, that is present on monocyes and neutrophils. The binding of P-selectin to the PSGL-1 ligand induces monocytes to secrete tissue factor, an agonist for the coagulation system. Upregulation of cytokine production is also observed when P-selectin binds the PSGL-1 receptor. As platelet-leukocyte and platelet-platelet aggregates circulate, there is potential for thrombosis, inflammation, and coagulation at distant sites.

Activated platelets may also shed microparticles, which are membrane-surrounded fragments of platelets that are < 1 µm in size. Microparticles may

Objective—To evaluate whether markers of platelet activation, including P-selectin expression, phosphatidylserine exposure, platelet-leukocyte aggregates, and microparticle formation, could be measured in nonstimulated and stimulated canine blood samples and develop a standardized protocol for detection of activated platelet markers in canine blood.

Sample population—Blood samples from 10 dogs.

Procedure—Platelet activation was determined by flow cytometric measurement of platelets with P-selectin expression, platelet-leukocyte aggregates, platelet microparticles, and platelets with phosphatidylserine exposure. Changes in specific markers of platelet activation in nonstimulated versus stimulated samples were assessed by use of varying concentrations of 2 platelet agonists, platelet-activating factor (PAF) and adenosine diphosphate. Flow cytometry was used to detect platelet CD61 (glycoprotein IIIa), CD62P (P-selectin), and the leukocyte marker CD45. Annexin V was used to identify exposed phosphatidylserine.

Results—A significant difference was detected in the percentages of platelets with P-selectin, platelet-leukocyte aggregates, microparticles, and platelets with annexin V exposure (phosphatidylserine) in samples stimulated with 10 nM PAF versus the nonstimulated samples, with platelet-leukocyte aggregates having the greatest difference.

Conclusions and Clinical Relevance—Platelet activation is essential for thrombus formation and hemostasis and may be potentially useful for evaluation of dogs with suspected thromboembolic disease. Prior to development of a thrombotic state, a prothrombotic state may exist in which only a small number of platelets is activated. Identification of a prothrombotic state by use of activated platelets may help direct medical intervention to prevent a thromboembolic episode.

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be functional, as they often have phosphatidylserine and platelet adhesive receptors, such as P-selectin and GPIIb/IIIa, on their surface. As microparticles circulate, they can interact with coagulation factors through the exposed phosphatidylserine, bind to leukocytes with P-selectin, or form platelet aggregates through their GPIIb/IIIa receptors. These interactions may contribute to inflammation and coagulation, possibly predisposing a patient to a thromboembolic event.15,16

A prothrombotic or hypercoagulable state may exist prior to a thromboembolic event in which a small amount of clot-promoting substance may cause thrombus formation, compared with a clinically normal patient. Although platelets only play a partial role in thromboembolic disease, measurement of their activation status may help detect a prothrombotic state and possibly a patient's predisposition for thromboembolic disease.17 Several platelet activation markers have been evaluated in human medicine to detect a prothrombotic state and include increased exposure of phosphatidylserine10 and P-selectin18 and increased numbers of platelet-leukocyte aggregates20-22 and platelet microparticles.20-22

Flow cytometry has been used to evaluate the activation state of platelets from humans and dogs. Most of the research completed in studies with platelets from dogs has been performed with PRP, platelets separated from plasma by cytocentrifugation and resuspended (washed platelets) or by gel filtration (gel-filtered platelets). These processes may permit in vitro platelet activation and often require a large volume of blood for detection of activated platelets in clinical patients. Detection of activated platelets in unprocessed blood from humans has been investigated. The advantages of using blood include the ability to use a small volume of blood and to potentially decrease in vitro platelet activation.21,22 The purpose of the study reported here was to evaluate whether markers of platelet activation, including P-selectin expression, phosphatidylserine exposure, platelet-leukocyte aggregates, and microparticle formation, could be measured in nonstimulated and stimulated canine blood samples and develop a standardized protocol for detection of activated platelet markers in canine blood.

Materials and Methods
Preliminary studies—Preliminary studies were performed to choose an anticoagulant for sample acquisition and antibodies and antibody concentrations for platelet markers, assess optimal antibody incubation time, and evaluate flow cytometry methods. On the basis of the best separation of RBC and platelet populations and the least amount of small particle formation, 3.2% sodium citrate was the anticoagulant chosen for this study (Figure 1). Antibodies were chosen on the basis of the largest separation of mean fluorescent intensity between the isotype control and cells with positive results for fluorescence. Prior to determining antibody concentrations, antibody incubation times between 20 and 45 minutes were evaluated. A 30-minute incubation time had the least amount of small particle formation and permitted adequate mean fluorescent intensity. Antibody saturation curves were performed to choose the optimal concentration of antibodies to use in each test. Finally, flow cytometry methods were assessed. Although the number of erythrocytes was greater than the number of platelets, coincidence count-

Dogs—Ten healthy client-owned dogs determined to be clinically normal via physical examination findings and results of CBCs were recruited for the study. Dogs had not received any medications within the last 6 months. Mean age of dogs was 5.1 years (range, 1 to 9 years). Six dogs were spayed females, and 4 were neutered males. Five were mixed-breed dogs. Breeds of the remaining 5 dogs included Labrador Retriever (n = 2), Border Collie (2), and Golden Retriever (1). Owners of dogs signed a client consent document permitting their dog to be used in the study, which was approved by the Washington State University Institutional Animal Care and Use Committee.

Sample collection—Venipuncture of a cephalic vein was performed with a 21-gauge butterfly needle. Four milliliters of blood was collected. The first 2 mL of blood was transferred to a tube containing EDTA for CBC. The next 2 mL of blood was drawn into a syringe containing 3.2% (0.11M) sodium citrate (1:9 ratio), which was used for platelet analysis.

Figure 1—Dot plots from preliminary studies performed for selection of an anticoagulant for detection of activated platelets in canine blood. A—Blood drawn directly into a syringe containing 3.2% sodium citrate (1:9 ratio). There are fewer small particles by use of 3.2% sodium citrate than EDTA. B—Blood drawn directly into a syringe containing EDTA. There are increased numbers of small particles, compared with the sodium citrate sample, but there is good separation between the platelet and RBC populations. SSC = Cell granularity. FSC = Size of cells.
In vitro agonist stimulation—A strong platelet agonist, PAF, in concentrations of 1 and 10nM and a weak platelet agonist, ADP, in concentrations of 10 and 20µM were used for platelet stimulation. Agonists were stored on ice until initiation of blood stimulation. In vitro agonist blood stimulation was performed within 15 minutes of obtaining blood samples. First, 90µL of blood was placed into 5 different wells in an ELISA 96-well plate, and then, 10µL of the specified agonist was added to the assigned sample well. Ten microliters of PBSS was added to the well containing the nonstimulated blood sample. Samples were covered with self-sealing film and incubated in a heating block at 37°C for 30 minutes to permit adequate platelet activation.

This study used all concentrations of PAF (1 and 10nM) and ADP (10 and 20µM) in samples evaluating platelets for P-selectin expression, phosphatidylserine exposure, and microparticle formation. Samples in which platelet-leukocyte aggregates were detected were stimulated with 10nM PAF and 20µM ADP.

Preparation of samples—After in vitro agonist stimulation, 5µL of stimulated or nonstimulated blood was added to specified polystyrene tubes containing a specific buffer, dependent on the specific monoclonal antibodies or markers used. After the addition of 5µL of blood to a specific buffer and monoclonal antibodies or markers, samples were incubated for 30 minutes at 37°C in the dark. Then, 500µL of 1% filtered, cold paraformaldehyde was added to each polystyrene tube and incubated at 4°C in the dark for 30 minutes. All stimulated and nonstimulated samples were further diluted in a ratio of 1:10 with filtered PBSS prior to analyzing with flow cytometry to decrease coincidence counting of erythrocytes. All buffers were filtered prior to use with a 0.20-µm syringe filter.

Detection of platelets—Platelets were recognized via flow cytometry by use of a monoclonal FITC or PE-conjugated anti-CD61 antibody against GPIIIa (CD61). A—The percentage of platelet microparticles in a nonstimulated blood sample was 3.8%. B—In a sample stimulated with 10nM PAF, the percentage of platelet microparticles has increased to 32.4% (arrow). Microparticles are considered platelets < 1 µm in size. FL2 = All platelet events. See Figure 1 for remainder of key.
detecting phosphatidylserine exposure, microparticles, and platelet-leukocyte aggregates. In the sample detecting P-selectin, 5 µL of a 1:2 dilution of anti-CD61 was the highest usable concentration that decreased antibody interference when platelets were dual labeled with the anti-CD62P monoclonal antibody. The anti-CD61 was diluted with filtered PBSS.

Detection of P-selectin—Detection of platelet surface P-selectin was performed by use of a monoclonal antibody, PE-conjugated anti-CD62P. Thirty microliters of anti-CD62P PE was determined via results of saturation curves to be adequate for detection of P-selectin in 5 µL of blood. Five microliters of nonstimulated or stimulated blood was added to 45 µL of PE-conjugated anti-CD62P. To help prevent binding interference between the anti-CD62P and anti-CD61 antibodies, the anti-CD62P was incubated alone with the blood sample for 2 minutes before 5 µL of a 1:2 dilution (diluted with filtered PBSS) of FITC-conjugated anti-CD61 was added.

Detection of phosphatidylserine—Detection of phosphatidylserine exposure was performed by use of FITC-conjugated annexin V, which is a calcium-dependent phospholipid-binding protein with high affinity for phosphatidylserine. First, 5 µL of nonstimulated or stimulated blood was added to 250 µL of annexin-binding buffer, which had been diluted in a ratio of 1:9 with distilled water. Then, 5 µL of annexin V-FITC and 10 µL of PE-conjugated anti-CD61 were added to the sample prior to incubation.

Detection of platelet-leukocyte aggregates—Platelet-leukocyte aggregates were detected by use of FITC-conjugated anti-CD61 and PE-conjugated anti-CD45, a panleukocyte marker. Results of saturation curves established 15 µL of anti-CD45 PE as the optimal concentration for detection of platelet-leukocyte aggregates. Five microliters of nonstimulated or stimulated blood was added to 10 µL of anti-CD61 antibody and 15 µL of anti-CD45 antibody in 475 µL of Hepes buffer (0.10mM Hepes, 0.133mM NaCl, 5.8mM KCl, 0.5mM glucose, and bovine serum albumin [1 mg/mL] in 100 mL of distilled water; pH, 7.4).

Controls—Isotype-matched controls for monoclonal antibodies against CD61, CD62P, and CD45 were used to detect nonspecific binding. Specifically, mouse IgG1 was used as an isotype control for the anti-CD61 monoclonal antibodies, rat IgG2a was used as an isotype control for the monoclonal antibody against CD45, and a PE-conjugated mouse IgG1 was used as an isotype control for P-selectin binding. The annexin-binding buffer was used as a negative control for the samples detecting phosphatidylserine exposure.

Flow cytometric analysis—After the processed blood samples had been fixed for 30 minutes and appropriately diluted, platelet analysis was evaluated by use of flow cytometry. Data were collected with a flow cytometer interfaced with a computer and analyzed via computer software. Background fluorescence was evaluated by use of samples containing isotype control antibodies or annexin-binding buffer, and gates were established so that approximately 99% of the events had no fluorescence. Compensation was performed for all dual-labeled samples to ensure adequate separation of events with and without fluorescence (positive and negative events, respectively). All platelet samples were gated by use of anti-CD61-positive events and all platelet-leukocyte samples were gated by use of anti-CD45 and anti-CD61 fluorescence, rather than gating solely by physical characteristics.

For detection of P-selectin expression in samples, 5,000 CD61-positive events were recorded with simultaneous collection of CD62P-positive events. Results were expressed as the percentage of CD61-positive events that were dual labeled with anti-CD62P. The samples detecting phosphatidylserine exposure were performed with the acquisition of 5,000 CD61-positive events and simultaneous collection of annexin V-positive events. The dual-labeled sample was expressed as the percentage of CD61-positive events that had exposure of phosphatidylserine. The percentage of microparticles in each sample was based on acquisition of 5,000 CD61-positive events. Platelet microparticles were defined as CD61-positive events < 1 µm in size. Platelets were distinguished from platelet microparticles by use of 1-µm latex beads and forward scatter characteristics. Microparticles were expressed as the percentage of CD61-positive events < 1 µm in size. To detect platelet-leukocyte aggregates, 2,000 CD45-positive events were recorded with simultaneous evaluation of CD61-positive events. The platelet-leukocyte aggregates were expressed as the percentage of the 2,000 CD45-positive events that were also labeled with a platelet marker (anti-CD61 antibody).

Statistical analysis—Computer software was used to perform all statistics. Data were analyzed by use of nonparametric Friedman 2-way ANOVA for detection of significant difference between the nonstimulated blood sample and the samples stimulated with the various agonists. A value of P < 0.05 was considered significant. A nonparametric t test, the Wilcoxon signed rank test, was used to assess significant differences between the

![Figure 4](image1)

Figure 4—Dot plots of P-selectin events. All platelets were gated by use of a fluorescent monoclonal antibody against CD61. A—In a nonstimulated blood sample, 0.1% of the platelet population has surface expression of P-selectin (arrow). B—In a sample stimulated with 10nM PAF, 5.0% of the platelet population has surface expression of P-selectin (arrow). See Figure 1 for remainder of key.
Results

A significant difference in the percentage of platelet microparticles (Figure 3), P-selectin events (Figure 4), platelet-leukocyte aggregates (Figure 5), and phosphatidylserine exposure (Figure 6) was observed between the nonstimulated blood samples and the blood samples stimulated with 10nM PAF in 10 dogs. Samples stimulated with ADP did not significantly differ from any of the platelet activation markers. The percentage of P-selectin–positive events in the nonstimulated sample ranged from 0% to 0.34% (mean ± SD, 0.1 ± 0.1%), compared with the sample stimulated with 10nM PAF, which ranged from 0.40% to 6.85% (2.8 ± 2.2%). The percentage of annexin V–positive events in the nonstimulated blood sample ranged from 1.1% to 7.3% (3.8 ± 1.9%), whereas the range for the sample stimulated with 10nM PAF was 7% to 47.3% (19.8 ± 14.4%). The percentage of platelet microparticles in the nonstimulated sample ranged from 1% to 4.7% (2.7 ± 1.1%), and the sample stimulated with 10nM PAF had a wide interval of 3.7% to 32.8% (12.2 ± 8.8%). Unlike the other 3 platelet activation markers, the percentages of platelet-leukocyte aggregates in the nonstimulated sample (1.6% to 12%; mean ± SD, 5.7 ± 3.8%) and the sample stimulated with 10nM PAF (22.7% to 66.6%, mean ± SD, 43.4 ± 13.7%) did not overlap.

After the experiment, anti-CD62P binding was reevaluated because of the low percentage of P-selectin–positive events detected in the experiment.
Anti-CD62P was used as a single label, and platelets were gated on their physical forward and side scatter characteristics to detect P-selectin expression in stimulated blood samples. As much as 20% to 30% of platelets in blood expressed P-selectin with 10nM PAF by use of a single label. However, when dual labeling was performed, the percentage of P-selectin expression decreased to approximately 3% to 7%.

**Discussion**

Thromboembolic events can be devastating in both humans and animals, causing a high risk of death. Detection of thromboembolism creates a diagnostic challenge, with cases of thromboembolism often diagnosed after death in veterinary medicine. In dogs, D dimers > 2,000 ng/mL have been reported as a specific indicator for the detection of thromboembolic disease.

However, a test that could measure the potential for a thromboembolic episode, or a prothrombotic state, may direct early medical intervention. Platelet activation is essential for thrombus formation and hemostasis and may be useful to evaluate in patients with a suspected prothrombotic or hypercoagulable state.

Our study evaluated whether 4 platelet activation markers could be measured in canine blood samples by use of flow cytometry. Two platelet agonists, ADP and PAF, were chosen for the study. In humans, ADP causes shape change, aggregation, and secretion of platelets in vitro, whereas PAF also causes shape change and aggregation. The variation of platelet activation in response to ADP appears to be species dependent. Results of a study comparing PAF- and ADP-stimulated platelets from various laboratory species indicate that canine platelets did not irreversibly aggregate in response to ADP, even with concentrations as high as 1,000 µM, whereas canine platelets irreversibly aggregated in response to PAF. In the study reported here, ADP did not have a significant stimulatory effect on blood samples, which supports previously published data. In contrast, there was a significant increase in the expression of P-selectin, annexin V, microparticles, and platelet-leukocyte aggregates after stimulation with 10nM PAF.

The baseline levels of P-selectin expression observed in our study were comparable to those in several studies performed with blood from humans. In a study using human blood, expression of P-selectin was detected in 1.1% of nonstimulated platelets. Other studies evaluating nonstimulated platelets in PRP from humans reported P-selectin expression ranging from 0.5% to 5%. The mean and median baseline levels of canine P-selectin expression in 2 studies with PRP were approximately 5% and 5.8%, respectively. In our study, the P-selectin expression of nonstimulated platelets ranged from 0.1% to 0.3%, which was different from results of a study in which P-selectin expression was detected in 20% to 25% of resting platelets in PRP from horses.

Mean P-selectin expression in our study after stimulation with 10nM PAF was 2.8%, which is lower than that in another study also performed in dogs. Few studies using platelets from humans or animals use PAF for blood platelet stimulation; however, 1 study using platelets in humans reported that approximately 25% of platelets expressed P-selectin after stimulation with 10nM PAF. Results of 2 studies with canine platelets in PRP and 10 ng/mL of phorbol myristate acetate as an agonist indicate that P-selectin expression is approximately 18% and 23%. Results of studies with platelets from humans indicate that P-selectin expression by use of PRP samples stimulated with ADP ranges from 17% to 60% when stimulated with 10 to 100 µM ADP and increased to as much as 92% when stimulated with a high dose of thrombin. Approximately 50% of equine platelets in a PRP preparation expressed P-selectin when activated with 1 U/mL of thrombin.

Initially, separate monoclonal antibody saturation curves were determined for the platelet marker (anti-CD61) and the P-selectin marker (anti-CD62P). Interaction of anti-CD61 and anti-CD62P antibody binding was detected during preliminary experiments with 10 µL of anti-CD61 antibodies. Because of these interactions, the lowest concentration of anti-CD61 that had adequate mean fluorescence intensity and still permitted anti-CD62P binding was chosen. The concentration of anti-CD62P used in our study, 30 µL, was chosen because of its adequate mean fluorescence intensity and its financial feasibility for use as a clinical diagnostic test. Therefore, this experiment was performed by use of 5 µL of a 1:2 dilution of anti-CD61 and 30 µL of anti-CD62P. Data acquired after the experiment supported continued interference with the chosen concentration of antibodies. For further studies, avoidance of antibody interaction may include use of a different platelet marker or a different P-selectin marker. In our study, little overlap was detected between nonstimulated and stimulated samples, suggesting that P-selectin may be a consistent marker of platelet activation. It is uncertain, however, whether it can be detected in a clinical patient by use of this protocol given the low percentage of P-selectin detected with 10nM PAF.

Platelet-leukocyte aggregates are another marker of platelet activation. Increases in platelet-leukocyte aggregates have been documented in human patients with sepsis, venous stasis, coronary disease, myeloproliferative disorders, and heparin-induced thrombocytopenia. Increased cytokine production, tissue factor expression, and increased numbers of cellular adhesion molecules are consequences of platelet-leukocyte binding, which suggests that communication between platelets and leukocytes may play a role in thrombosis and inflammation. Binding of platelets to leukocytes could also permit platelets to remain in circulation for a longer duration and serve as an ongoing marker of platelet activation.

The baseline percentage of platelet-leukocyte aggregates in blood from humans ranges from 1.76% to 3%. In our study, the mean percentage of platelet-leukocyte aggregates in nonstimulated samples was 5.7%, which is slightly higher than that reported in the medical literature from humans. Increasing concentrations of ADP cause the percentage of platelet-leukocyte aggregates in blood from humans to increase by as much as 47% when stimulated with 10 µM ADP. Stimulation with ADP did not cause a significant
change in the percentage of platelet-leukocyte aggregates in our canine samples, whereas PAF caused an increase in platelet-leukocyte aggregates similar to what is reported in the human medical literature with ADP. Given the consistent difference between nonstimulated and stimulated canine blood samples in our study, platelet-leukocyte aggregates may be useful as a diagnostic test to evaluate blood samples for activated platelets. The major disadvantage noted in our study was the time required for evaluation of a sample because of the low numbers of leukocytes, compared with platelets and RBCs in whole blood samples.

A third activated platelet marker is phosphatidylserine, which can be detected with annexin V binding. In our study, a significant difference in phosphatidylserine exposure was detected between the nonstimulated sample and the sample stimulated with 10nM PAF. Results of another study with canine platelets also indicated an increase in annexin V binding with 10nM PAF as an agonist. Results of a study using equine PRP indicate that phosphatidylserine exposure increases after use of 10nM PAF but not ADP. In the study reported here, although a significant difference in phosphatidylserine exposure was detected, the extreme variability in annexin V expression (19.5 ± 14.5%) could make interpretation difficult if used in a clinical setting.

The percentage of microparticles in the nonstimulated sample in our study was < 5%, which was comparable to results of another study in which 5% of the platelet population consisted of microparticles in nonstimulated PRP samples from dogs. The percentage of platelet microparticles increased significantly in our study when exposed to 10nM PAF, with as much as 32.8% of the platelet population consisting of platelet microparticles in 1 dog and a mean of 12.2% in all 10 dogs. Approximately 40% microparticles in PRP with thrombin stimulation has been reported. Platelet microparticles were easy to detect and could be rapidly acquired, suggesting that this platelet activation marker may be useful in a clinical setting.

Results with 3 of the 4 platelet markers, including platelet-leukocyte aggregates, microparticles, and phosphatidylserine exposure, appeared to parallel each other. The baseline percentages of these activation markers were similar, approximately 3% to 5%, and there was a 4- to 8-fold increase in detection of the activation markers after stimulation with 10nM PAF. The mean percentage of P-selectin was low in the nonstimulated sample, which was not comparable to the other 3 markers. This discrepancy was concerning because it was evident that P-selectin was expressed to permit platelet-leukocyte aggregate formation. The low detection rate of P-selectin with 10nM PAF was also inconsistent with results of the other 3 activation markers. Antibody interference was detected, but it is not certain whether this was the only contributor. Another consideration includes loss of P-selectin from the membrane because P-selectin is rapidly shed from the membrane surface after activation. Results of 1 study indicate a 75% decrease in platelets with P-selectin 15 minutes after platelet activation. A third consideration is that the antibody was unable to detect P-selectin deep within platelet-leukocyte aggregates. Detection of P-selectin in blood with the protocol used in the study reported here may be less sensitive, compared with other methods of P-selectin evaluation, primarily by use of PRP because of the low numbers of P-selectin events detected in the study. However, the techniques and antibodies differed between experiments, and further studies are needed to clarify the clinical use of P-selectin detection in blood.

In our study, the choice of anticoagulant was determined on the basis of results of preliminary studies evaluating separation of RBCs and platelet populations and the number of small particles. The small particles were composed of low numbers of labeled platelets, platelet microparticles, and probable debris. Our results indicated that 3.2% sodium citrate caused fewer numbers of small particles. Results of another study indicate that EDTA is better than 3.8% sodium citrate for platelet activation studies because EDTA caused less ex vivo platelet activation for at least 4 hours. In our study, the samples were evaluated quickly, within 15 minutes of obtaining a blood sample; therefore, a difference between platelet activation in EDTA and 3.2% sodium citrate was probably not detected. However, use of 3.2% sodium citrate and the rapid time frame that platelet activation studies were performed could potentially limit the usefulness of this technique as a clinical test.

Our results indicated that platelet activation markers can be measured in canine blood samples stimulated with 10nM PAF by use of flow cytometry. Use of platelet activation markers for diagnostic testing requires that the protocol presented here be used with little deviation and that the test be performed soon after obtaining a blood sample to decrease in vitro platelet activation. Further evaluation of P-selectin antibodies and protocols should be performed to further optimize this test, as a greater separation between nonstimulated and stimulated blood samples would be optimal. Different protocols with annexin V can also be evaluated to decrease the extreme variation detected in our study. Reference intervals also need to be established by use of a large number of clinically normal dogs.

In human medicine, P-selectin expression and platelet-leukocyte aggregates are considered the most sensitive tests for platelet activation, although there is debate on which of these tests is the best overall. Our results suggested that detection of platelet-leukocyte aggregates is the most sensitive and reliable marker of platelet activation in canine blood and that evaluation of platelet microparticles may also be useful. Future clinical studies evaluating platelet activation in clinically ill patients may help evaluate which platelet activation markers will be the most valuable.

b. Parafilm, Sigma-Aldrich, St Louis, Mo.
c. Clone RUU-PL7F12, 0.0125 ng/mL, batch No. 53077, Pharmingen, Becton Dickinson, San Diego, Calif.
d. Clone VI-PL2, 0.0125 ng/mL, batch No. 43196, Pharmingen, Becton Dickinson, San Diego, Calif.
e. Clone AG. 1.250 µg/mL, lot No. 43634, Immunocytochemistry Systems, Becton-Dickinson, San Jose, Calif.
f. Annexin V-FITC, lot No. 75530, Pharmingen, Becton-Dickinson, San Diego, Calif.
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