

# Alterations in Normal Canine Platelets During Storage in EDTA Anticoagulated Blood

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**Abstract:** Flow cytometric detection of platelet surface-associated IgG (PSAIgG) can be used to determine whether immunologic factors are contributing to thrombocytopenia in dogs. In vitro alterations in platelet activation and morphology, however, could impact the results of this test. The purpose of this study was to determine whether the PSAIgG test for immune-mediated thrombocytopenia was valid on whole blood in EDTA anticoagulant after 24-72 hours of storage, and to characterize other alterations in canine platelets that could impact immunologic testing. Platelets were harvested and analyzed immediately after blood collection and after 24, 48, and 72 hours of storage at 4°C. Spontaneous and thrombin-induced changes in the following platelet parameters were evaluated using flow cytometric techniques: PSAIgG, platelet microparticle formation, membrane expression of P-selectin and glycoprotein CD61, exogenous IgG binding, surface-exposed phosphatidylserine, and fibrinogen binding. The amount of PSAIgG increased 6- to 9-fold in stored samples compared with fresh samples. Platelet microparticle formation was spontaneous in stored samples and increased significantly over time. Membrane phosphatidylserine, P-selectin, and fibrinogen binding were not altered by storage, indicating that platelet activation was minimal in stored samples. Although storage decreased the percentage of platelets positive for CD61 by 8- to 10-fold compared with fresh samples, activation by high-dose thrombin partially restored the percentage of CD61-positive platelets in 24-hour-old samples. In conclusion, even though platelets stored in EDTA for up to 72 hours remain in a resting state, aged platelets have an increased tendency to form microparticles and have increased surface IgG and decreased surface CD61, which may contribute to false-positive results for tests of immune-mediated thrombocytopenia. (*Vet Clin Pathol.* 2001;30:107-113) ©2001 American Society for Veterinary Clinical Pathology

**Key Words:** CD61, fibrinogen, microparticles, phosphatidylserine, platelet activation, PSAIgG, P-selectin

Platelets undergo a variety of changes during activation; the calcium chelators in anticoagulants halt these alterations. Less well known are the changes platelets undergo in vitro as they age in anticoagulant solutions. For example, platelet microparticles form by budding from membranes either spontaneously in stored platelet concentrates or during platelet activation.<sup>1-6</sup> Platelet microparticles are <1 µm in diameter, in contrast to whole platelets, which are 2-4 µm in size.<sup>7</sup> Despite their small size, platelet microparticles have procoagulant activity and are rich in platelet factor 3.<sup>8</sup> Redistribution of phosphatidylserine from the inner membrane surface to the exterior surface accompanies microparticle formation during platelet activation.<sup>8</sup> Exposed phosphatidylserine at the platelet surface is subsequently involved in assembly of the prothrombinase complex.<sup>9</sup> Upon activation, platelets release endogenously synthesized pro-

teins from alpha granules. These proteins, including P-selectin and von Willebrand factor, are redistributed to the outer platelet membrane where they are important for adherence to damaged subendothelium and leukocytes.<sup>10</sup> Redistribution of internal stores of fibrinogen and CD41/CD61 to the outer plasma membrane surface also occurs during platelet activation.<sup>11</sup> Later in the formation of a clot, plasma fibrinogen binds the CD41/CD61 integrin complex and serves as a bridge between platelets.

Data on the stability of resting platelets in stored canine blood samples are limited. It is not known how morphologic changes that occur with storage affect interpretation of the PSAIgG test for canine immune-mediated thrombocytopenia. Resting platelets can internalize exogenous proteins such as fibrinogen, IgG, fibronectin, and albumin from the plasma into alpha

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granules by receptor-mediated endocytosis.<sup>12</sup> The accumulation of intracellular fibrinogen and IgG occurs in mature canine platelets during storage *in vitro*.<sup>13</sup> Redistribution of internalized proteins to the surface by eversion of the alpha granules into the open canicular system could cause false-positive results for tests measuring PSAIgG on stored samples.

Flow cytometric assays to detect PSAIgG in fresh canine platelets are rapid and have high specificity and sensitivity compared with other methods.<sup>14-16</sup> Previous flow cytometric studies suggested that EDTA-anticoagulated blood samples for PSAIgG testing could be stored at refrigeration temperatures up to 72 hours prior to analysis without causing false-positive test results.<sup>17</sup> The purpose of this study was to determine whether the PSAIgG test for immune-mediated thrombocytopenia was valid after 24-72 hours of storage of whole blood in EDTA anticoagulant. We hypothesized that PSAIgG levels would increase during platelet storage. In addition, alterations in fibrinogen binding, platelet microparticle formation, and platelet surface expression of CD61, P-selectin, and phosphatidylserine were compared in fresh and stored samples under resting and activated conditions using flow cytometric techniques. The results of this study will help characterize platelet alterations in stored samples that may impact the results of diagnostic testing for immune-mediated thrombocytopenia.

### Materials and Methods

#### Blood and platelet samples

Blood was collected in K<sub>3</sub>EDTA (Vacutainer, Becton Dickinson, San Jose, Calif, USA) from 4 healthy adult purpose-bred Greyhound dogs housed according to Animal Care and Use regulations of the National Institutes of Health.

Platelet-rich plasma was prepared immediately as described previously<sup>17</sup> from a 4-mL aliquot of whole blood and was analyzed within 4 hours of collection. Remaining whole blood samples were stored at 4°C and processed 24, 48, and 72 hours after collection. Stored whole blood samples were allowed to warm to room temperature prior to preparation of platelet-rich plasma. Platelet-rich plasma was obtained by centrifugation at room temperature for 80 seconds at 800g (GCL-1 Sorval, General Laboratory Centrifuge, Newton, Conn, USA). Platelets were washed 3 times in modified Tyrode's buffer<sup>17</sup> (nonstimulation buffer containing 0.14 M NaCl, 0.003 M KCl, 0.005 M NaH<sub>2</sub>PO<sub>4</sub>, 0.001 M NaHCO<sub>3</sub>, 0.006 M Na<sub>2</sub>EDTA, pH 7.1; Sigma Chemical Co, St. Louis, Mo, USA) to remove plasma proteins. Platelets were resuspended in Tyrode's buffer to a concentration of  $1 \times 10^7$  cells/100  $\mu$ L (10,000 cells/mL).

#### Platelet analyses

Platelets were diluted to 100,000 cells/ $\mu$ L in Tyrode's buffer. A 100  $\mu$ L sample of washed platelet suspension was added to 100  $\mu$ L of buffer or reagent (prepared in Tyrode's buffer with 1% bovine serum albumin; Sigma) in plastic tubes (Falcon #2054, Becton Dickinson) for each of the assays.

*Platelet microparticles.* Platelets were added to Tyrode's buffer for flow cytometric analysis of platelet size.

*PSAIgG.* To detect bound IgG, platelets were added to monoclonal antibody (mAb) specific for canine IgG (final concentration of 24  $\mu$ g/mL; Sigma) conjugated to the green dye fluorescein isothiocyanate (FITC). A second sample containing FITC-conjugated isotype-specific mouse IgG1 (24  $\mu$ g/mL; Sigma) was used to detect nonspecific binding of mAb.

*CD61.* To determine expression of the platelet glycoprotein CD61, platelet suspension was added to FITC-anti-CD61 antibody (0.63  $\mu$ g/mL, RUU-PL7F12, Becton Dickinson).<sup>4</sup>

*Fibrinogen binding.* To assess fibrinogen binding to the platelet surface, human fibrinogen conjugated to the green dye Alexa (10  $\mu$ g/mL, Molecular Probes, Eugene, Oreg, USA) was admixed with platelet suspension.

*P-selectin.* For detection of P-selectin on the platelet surface, mAb G5 (10  $\mu$ L, Dr R. McEver, Oklahoma Medical Research Foundation, Oklahoma City, Okla, USA) was first incubated with 100  $\mu$ L platelet suspension, followed by 3 washes in Tyrode's buffer, and a second incubation with FITC-conjugated anti-mouse IgG (H+L) (Bethyl Labs, Montgomery, Tex, USA). In a separate tube, the secondary antibody was added to an aliquot of platelet suspension without primary antibody to P-selectin; this sample was analyzed for nonspecific binding using indirect immunofluorescent flow cytometry. After mixing and a 30-minute incubation in the dark at room temperature, samples were washed 3 times in Tyrode's buffer and immediately analyzed by flow cytometry.

*Phosphatidylserine.* To detect phosphatidylserine on the external platelet membrane, 5  $\mu$ L of Annexin V-FITC (PharMingen, San Diego, Calif, USA) was added to 100  $\mu$ L of platelet suspension and incubated for 15 minutes followed by 400  $\mu$ L of either Tyrode's buffer or binding buffer containing 0.01 M HEPES buffer/NaOH, pH 7.4, 0.14 M NaCl, and 2.5 mM CaCl<sub>2</sub>.

*Binding of exogenous IgG.* To determine whether fresh and stored platelets bound exogenous IgG, 100  $\mu$ L of FITC-conjugated mouse IgG1 (final concentration of 24  $\mu$ g/mL) was added to 100  $\mu$ L of EDTA-treated whole blood containing  $\sim 3 \times 10^6$  platelets. Platelets were isolat-

ed from the whole blood samples following incubation for 4, 24, 48, or 72 hours. Plasma proteins and unbound FITC-labeled IgG were washed free of the platelet concentrate prior to analysis by flow cytometry.

### Platelet activation studies

The effect of storage on platelet activation was determined by measuring differences in PSAIgG, P-selectin, and fibrinogen binding following stimulation with ionomycin (a calcium ionophore that stimulates cells through mobilization of intracellular calcium stores independent of membrane receptor binding) and thrombin. Approximately  $10^7$  washed platelets were resuspended in 1 mL of each of the following: Tyrode's buffer, 1 mM  $\text{CaCl}_2$  buffer with ionomycin (6  $\mu\text{M}$ ), 1 mM  $\text{CaCl}_2$  buffer with 0.2 U/mL bovine thrombin, or 1 mM  $\text{CaCl}_2$  buffer with 2.0 U/mL thrombin (Sigma).

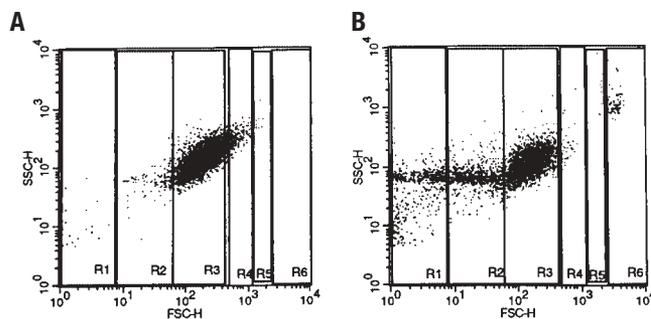
### Flow cytometric analysis

Platelet reactions with each fluorescent antibody or protein (fibrinogen and Annexin-V) were determined using flow cytometric analysis. Autofluorescence was determined using unlabeled platelets. Background fluorescence was established using samples stained with isotype control antibody (FITC-labeled mouse IgG1) or secondary antibody (FITC-labeled anti-mouse IgG H+L) in which >95% of fluorescent events were within the negative channels of the histogram. Except for the Annexin-V assay, results of all analyses were expressed as the percentage of platelets that shifted into the positive fluorescent channels (FL1) of the histogram. Phosphatidylserine membrane exposure or binding by Annexin-V was recorded as the mean shift in fluorescence of the positive channels of the histogram.

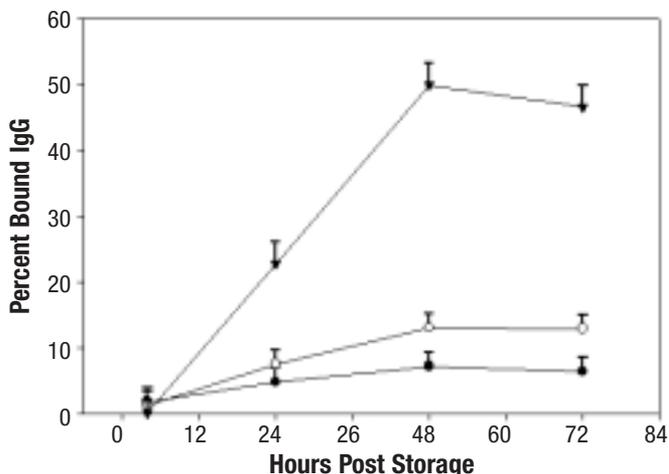
Data were analyzed with a Becton Dickinson FACScan equipped with a 488-nm argon laser interfaced with a 7600 Power Macintosh computer and Cell Quest software (Becton Dickinson). The electronic log settings for platelet analysis were forward light scatter (FSC) voltage=E00, AmpGain=2.0, side light scatter (SSC) voltage=346, green fluorescence detector voltage (FL1)=742, SSC threshold=170. Normal platelets (1-3  $\mu\text{m}$ ) were distinguished from microparticles (<1  $\mu\text{m}$ ) and RBCs (>6  $\mu\text{m}$ ) by gating for size. Gates to distinguish different sizes of platelets were based on the FSC properties of latex beads ranging in size from 0.1 to 9.0  $\mu\text{m}$  (Polybead polystyrene, Polysciences, Warrington, Penn, USA) (Figure 1).

### Statistical analysis

Data were analyzed by ANOVA for a split plot design



**Figure 1.** Dot plots of side scatter (SSC-H) and forward scatter (FSC-H) properties of fresh normal platelets (A) and 48-hour-old platelets, which include platelet microparticles (B). Each region (R) corresponds to a diameter size range based on light scatter properties of latex beads: R1=0.1-0.5  $\mu\text{m}$ ; R2=0.5-1.4  $\mu\text{m}$ ; R3=1.4-3.0  $\mu\text{m}$ ; R4=3.0  $\mu\text{m}$ ; R5=6  $\mu\text{m}$ ; R6=9-10  $\mu\text{m}$ .



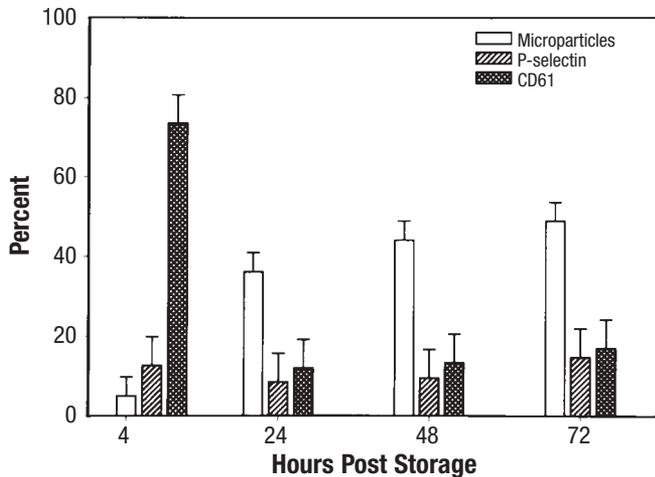
**Figure 2.** Effect of storage on PSAIgG values and exogenous IgG binding. Solid circles indicate the percentage of platelets non-specifically reacting with FITC-conjugated mouse IgG1 added to platelet concentrates harvested at defined storage times. Open circles indicate the percentage of platelets with bound PSAIgG. Closed triangles indicate the percentage of platelets binding FITC-conjugated mouse IgG1 added to whole blood and incubated for up to 72 hours. Data are the mean  $\pm$  SEM of 2 experiments.

that included treatment  $\times$  hour effects. Effects were considered significant at  $P < .01$ .

## Results

### Effect of storage on platelet parameters

The percentage of platelets with detectable PSAIgG increased from 1.4% in 4-hour samples to 8% in 24-hour samples and 13% in samples stored for 48 and 72 hours ( $P < .01$ ) (Figure 2). In contrast, there was no significant increase in nonspecific binding by the irrelevant isotype control antibody to stored platelets ( $P < .10$ ). IgG was not



**Figure 3.** Effect of storage on the percentage of platelet microparticles, P-selectin-positive platelets, and CD61-positive platelets. Data are mean  $\pm$  SEM of 2 experiments.

detected on platelets 4 hours after addition of excess FITC-labeled mouse IgG1 to whole blood (Figure 2). However, fluorescent labeled IgG1 was detected on platelets analyzed 24, 48, and 72 hours after whole blood incubation with exogenous IgG.

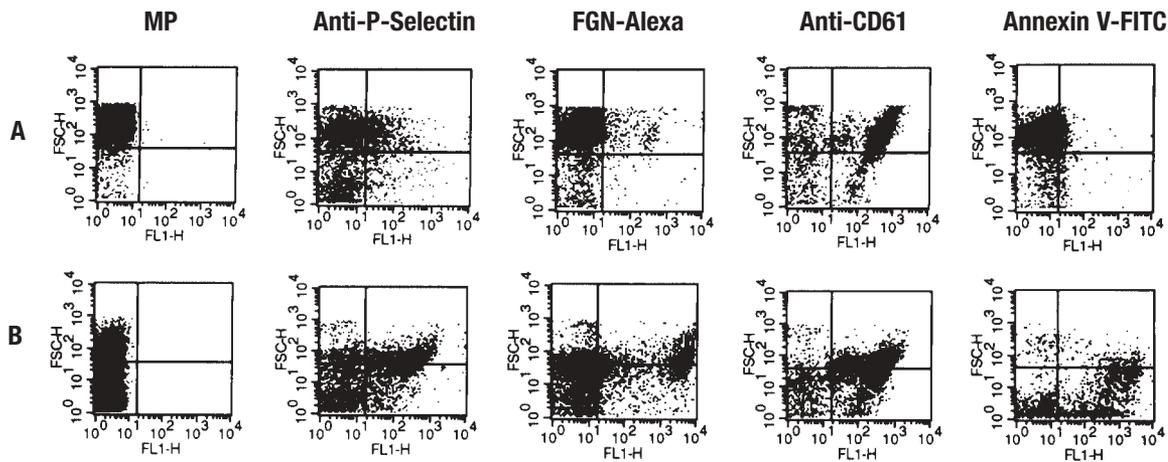
Microparticles increased 7- to 10-fold after 24 to 72 hours of storage ( $P < .001$ ) (Figure 3). There was no significant change in the percentage of platelets positive for

P-selectin regardless of the duration of storage ( $P < .07$ ) (Figures 3, 4). The percentage of CD61-positive platelets was highest in fresh platelet samples (mean, 73%), whereas the percentage of platelets positive for CD61 decreased to  $<20\%$  following storage ( $P < .001$ ). The decrease in CD61 expression corresponded with increased platelet microparticle formation. Phosphatidylserine exposure on the membrane of unstimulated platelets also was minimal and was not affected by storage ( $P = .1$ ) (Figure 5).

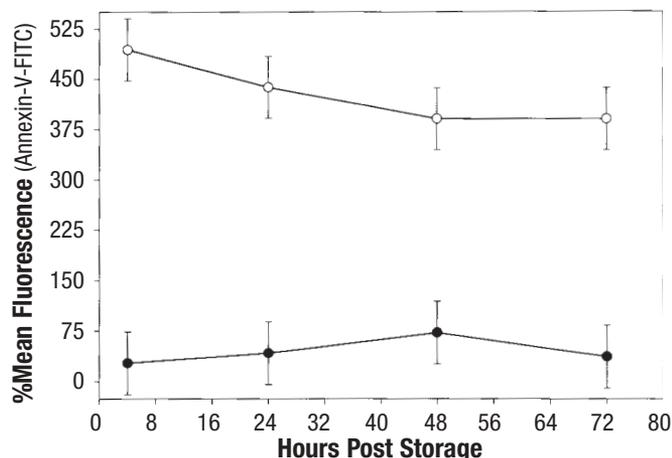
Effect of activation on platelet parameters

Under resting conditions, P-selectin and fibrinogen were detected on only a small percentage of platelets, suggesting minimal activation subsequent to either the washing steps or the application of labeled proteins (Figure 4). An increased amount of surface P-selectin, fibrinogen, and microparticle formation was detected on activated platelets compared with resting platelets. Activation also induced a 10-fold increase in Annexin-V binding to exposed phosphatidylserine in fresh samples, which remained high (8-fold) following 24, 48, and 72 hours of storage ( $P < .001$ ) (Figure 5). Furthermore, activation caused increased expression of P-selectin, CD61, and fibrinogen on platelet microparticles.

Ionomycin caused an increase in P-selectin and fib-



**Figure 4.** Bivariate dot plots depicting forward scatter (size, FSC-H) and green fluorescence (FL1-H) for resting platelets (A) and platelets activated with 2.0 U of thrombin (B). MP indicates platelet microparticles, anti-P selectin detects surface P-selectin, FGN-Alexa detects surface-bound fibrinogen, anti-CD61 detects surface CD61, and Annexin V-FITC detects surface-exposed phosphatidylserine. Signals in the upper and lower left quadrants represent FL1-negative cells. Signals in the lower left and lower right quadrants represent platelet microparticles ( $<1.0 \mu\text{m}$  in diameter). Signals in the upper right and lower right quadrants together represent the FL1-positive cells for each parameter. Unstained platelets of normal size are in the upper left quadrant, and platelet microparticles are in the lower left quadrant. The shift in fluorescence on the FL1-H axis reflects the percentage of normal-size platelets (upper right quadrant) or platelet microparticles (lower right quadrant) bound to the fluorescent label. The number of platelet microparticles was increased under activated (B) compared with resting (A) conditions. The amount of surface P-selectin, bound fibrinogen, and phosphatidylserine also was greater in activated platelets (and in microparticles) than in resting platelets.



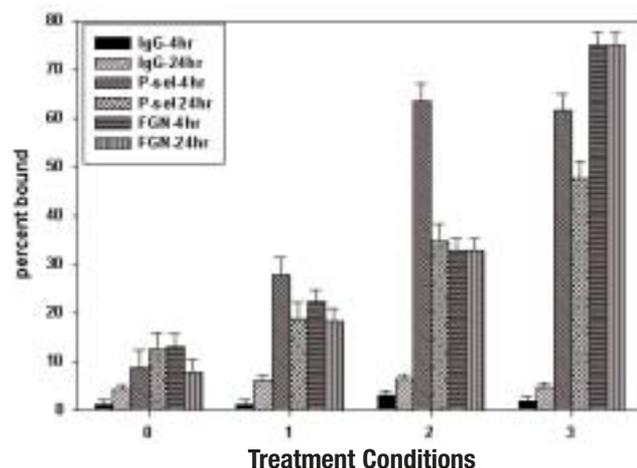
**Figure 5.** Phosphatidylserine exposure on the platelet surface of resting platelets (solid circles) and platelets activated with calcium-binding buffer (open circles). Data are the mean  $\pm$  SEM of 2 experiments.

rinogen binding above resting levels ( $P < .001$ ) but less than that with thrombin treatment ( $P < .001$ ) (Figure 6). In fresh platelet samples, P-selectin and fibrinogen binding increased with thrombin concentration. However, the percentage of platelets positive for P-selectin in 24-hour-old samples treated with either ionomycin or thrombin was less than that in 4-hour samples ( $P < .01$ ). The 24-hour-old platelets were still capable of binding fibrinogen to the same degree as were fresh platelets following activation with both agonists. Thrombin stimulated more binding by fibrinogen than did ionomycin, and this binding was dose-dependent.

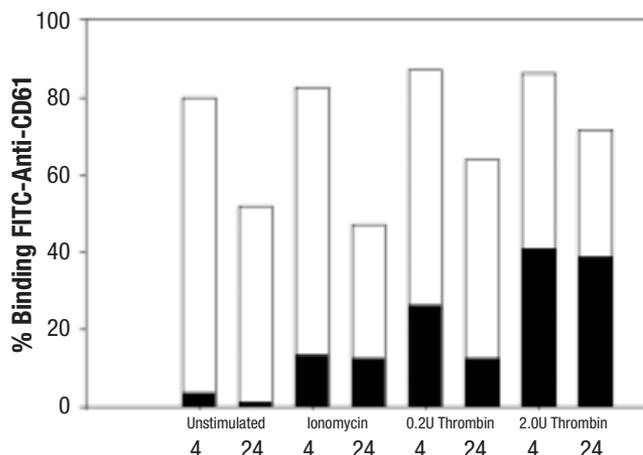
The percentage of platelets with PSAIgG did not increase significantly over resting levels following activation of fresh samples, and increased only minimally when stimulated with ionomycin or 0.2 U of thrombin ( $P < .05$ ) in 24-hour-old samples (Figure 6). PSAIgG values in 24-hour-old platelets stimulated with low doses of thrombin increased to levels similar to those detected for 24-hour-old nonstimulated platelets, but were lower than those for nonstimulated 24-hour samples incubated with exogenous mouse IgG1.

Eighty percent of fresh platelets were positive for CD61, regardless of stimulation conditions (Figure 7). After 24 hours of storage the proportion of CD61-positive platelets decreased to 45% in unstimulated samples. A similar response was demonstrated earlier (Figure 4). Activation with 2 concentrations of thrombin (0.2 and 2.0 U) but not ionomycin partially restored the percentage of CD61-positive platelets in stored samples to values observed at 4 hours ( $P < .001$ ) (Figure 7).

Platelet microparticles were scarce in 4- and 24-hour-old resting samples. However, the percentage of



**Figure 6.** Effect of treatment conditions (0=nonstimulation buffer, 1=6  $\mu$ M ionomycin, 2=0.2 U thrombin, and 3=2.0 U thrombin) on the percentage of platelets with surface IgG, P-selectin (P-sel), and bound fibrinogen (FGN) after 4 and 24 hours of storage. Data are the mean  $\pm$  SEM of 3 experiments. Activation of freshly prepared canine platelets with platelet agonists increased the expression of P-selectin and fibrinogen binding.



**Figure 7.** Effects of activation and storage for 4 or 24 hours on the expression of CD61 on whole platelets (open bars) and platelet microparticles (solid bars). Data are the mean  $\pm$  SEM.

platelet microparticles positive for CD61 increased with activation and reached 40% of the total platelet population when activated with high doses of thrombin, irrespective of storage time (Figure 3).

### Discussion

In this study, we observed several alterations in canine platelets stored in EDTA. Canine platelets underwent changes similar to those described for stored human platelets.<sup>2,3</sup> Increased IgG on the surface of platelets is thought to indicate an immunologic mechanism of thrombocytopenia.<sup>20-22</sup> However, as determined here,

the age of the blood sample could affect interpretation of increased PSAIgG values. The percentage of platelets with PSAIgG increased in whole blood samples that were stored 24 hours or longer. Based on the findings in this and another study,<sup>13</sup> we hypothesize that plasma IgG may be internalized by canine platelets during storage and then redistributed to the surface. This assumption is based on 2 observations. First, little nonspecific binding of FITC-conjugated mouse IgG1 occurred when a fluorescent IgG molecule was added directly to platelet concentrates from stored blood. Second, binding of exogenous IgG1 was not detected in platelets harvested from whole blood until after the blood was incubated with FITC-labeled IgG1 for  $\geq 24$  hours. If the increased IgG on the surface of platelets was due to binding of the IgG molecule to Fc receptors, PSAIgG should be detected immediately after addition of the molecule. These observations support those in another study in which IgG and fibrinogen accumulated on stored platelets that were 4-5 days old but not on fresh platelets.<sup>13</sup>

Resting canine platelets shed microparticles  $< 1.0 \mu\text{m}$  in diameter following storage and aging. Microparticle formation is known to increase with activation of canine platelets,<sup>4</sup> however, this is the first report of platelet microparticle formation in aged normal canine platelets. Platelet microparticles express prothrombinase complex (factors Xa, Va, and  $\text{Ca}^{2+}$ ) binding sites that are rich in acidic phospholipid, resulting from transbilayer migration of phosphatidylserine from the inner to the outer leaflet of the bilayer.<sup>5</sup> High concentrations of platelet microparticles also can be thrombogenic in certain clinical disorders.<sup>23</sup> Impaired hemostasis in patients with immune-mediated thrombocytopenia has been associated with anti-phospholipid antibodies that have a high affinity for platelet microparticles.<sup>24</sup> In the current study, canine microparticles developed in resting platelets aged in vitro, and were generated after activation. Furthermore, canine P-selectin, CD61, phosphatidylserine, and fibrinogen binding sites were detected on platelet microparticles.

The percentage of CD61-positive platelets decreased in stored samples. Although platelet microparticle formation increased in stored samples, shedding of CD61 on platelet microparticles was minimal in unstimulated samples and did not explain the decrease in CD61 expression. Moreover, activation with strong agonists such as thrombin partially restored the CD61 on stored platelets. Detection of CD61 is dependent on the ability of the antibody to recognize epitopes specific to CD61 and not epitopes produced by the calcium-dependent conformation of the CD41/CD61 complex.<sup>25,26</sup> Therefore, the decrease in CD61 expression following storage in EDTA may have been due to internalization of the glycoprotein by endocytosis<sup>27,28</sup> rather than a

result of dissociation of the calcium-dependent configuration of the CD41/CD61 complex following calcium chelation. The partial restoration of anti-CD61 antibody binding after stimulation with platelet agonists may be explained by redistribution of the CD61 glycoprotein from the alpha granule membranes to the surface membrane.<sup>11</sup>

Although stored platelets bound fibrinogen, had surface-exposed phosphatidylserine, and formed platelet microparticles following either ionomycin or thrombin stimulation, surface P-selectin expression induced by either agonist was less in 24-hour-old samples than in freshly activated platelets. Similar findings were reported in which P-selectin responses to thrombin were impaired in canine platelets that were 3, 4, and 5 days old.<sup>29</sup> Possible mechanisms include the loss of functional thrombin receptors from aged platelets or an age-related alteration in the second messenger system for the thrombin receptor.<sup>29</sup>

Canine platelets stored in vitro in EDTA anticoagulant tend to have mild increases in PSAIgG and platelet microparticles and decreased surface CD61 but still have the ability to undergo activation. Accurate interpretation of PSAIgG values in stored blood samples from thrombocytopenic patients requires understanding of the background levels of PSAIgG in normal canine samples handled in the same manner.  $\diamond$

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