A vital function of platelets is to adhere to a site of vascular injury where collagen and other extracellular matrix proteins, including tissue factor, are exposed to flowing blood. The platelets provide a cellular surface for localized thrombin generation. In the past, platelets were considered a homogenous population with equal potential for activation, which would lead to stimulation of local generation of thrombin. However, recent evidence suggests that all activated platelets are not equal and that a subset (ie, coated platelets) that can be observed after combined stimulation with dual agonists (ie, collagen and thrombin) have a unique potential for retaining procoagulant activity on their surface.1

Coated platelets are defined as platelets with high concentrations of α-granule proteins, including fibrinogen, von Willebrand factor, thrombospondin, and coagulation factor V,2 retained with exceptional affinity.

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Abbreviations

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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>APC</td>
<td>Allophycocyanin</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FU</td>
<td>Fluorescence unit</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>PerCP</td>
<td>Peridinin-chlorophyll-protein complex</td>
</tr>
<tr>
<td>rhFVIIa</td>
<td>Active site–inhibited recombinant human coagulation factor VIIa</td>
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were considered a homogenous population with equal potential for activation, which would lead to stimulation of local generation of thrombin. However, recent evidence suggests that all activated platelets are not equal and that a subset (ie, coated platelets) that can be observed after combined stimulation with dual agonists (ie, collagen and thrombin) have a unique potential for retaining procoagulant activity on their surface.1

Coated platelets are defined as platelets with high concentrations of α-granule proteins, including fibrinogen, von Willebrand factor, thrombospondin, and coagulation factor V,2 retained with exceptional affinity.
on their surfaces via serotonin-mediated coupling by means of transglutaminase activity. Physiologic agonists of platelets (eg, ADP, which is widely available in platelets and erythrocytes) are able to activate platelets as assessed by use of traditional markers of platelet activation (eg, P-selectin) and to increase the amount of fibrinogen binding, although this binding is reversible and is only at a low degree. Uniquely, formation of a subpopulation of platelets with the coated phenotype (eg, characterized by high fibrinogen binding) requires the combined stimulation of thrombin and collagen or convulxin. Even when maximal externalization of phosphatidylserine on all platelets was induced via nonphysiologic stimulation with an ionophore, only a subpopulation of platelets had the coated phenotype with high externalization of \( \alpha \)-granule protein. The enhanced procoagulant potential of coated platelets is used in the pharmacological application of recombinant human coagulation factor VIIa, which binds preferentially to coated platelets.

The complexity of platelet activation and platelet-platelet interactions complicates research on platelet physiologic processes and platelet-related disease. This poses a problem with regard to diagnosis in veterinary medicine and also in translational research that is intended to use knowledge from naturally occurring diseases in dog populations as a means for studying diseases in humans. Several relevant diseases (eg, hemophilia A and B, coagulation factor VII deficiency, von Willebrand disease, Glanzmann thrombasthenia, platelet dense \( \alpha \) granule defect, and Scott syndrome) exist in dogs. Furthermore, undiagnosed platelet defects in dogs, some of which may be relevant to diseases in humans, may be identified through the use of more advanced biomolecular tools.

To correctly characterize platelet disorders and accurately assess the procoagulant potential of canine platelets, a broader panel of surface markers for platelet activation is needed. Such a panel would potentially allow detection of extremely subtle differences in platelet activation. Traditionally, expression of P-selectin on the surface of platelets has been used as a measure of platelet activation. Stimulation of canine platelets with a single agonist has been used for assessment of maximal platelet expression of P-selectin. For human platelets, no distinct patterns for expression of P-selectin were identified in experiments that revealed differential binding of coagulation factors to coated and noncoated platelets. Therefore, more subtle distinctions between P-selectin expression and additional markers of platelet activation may facilitate a better understanding of the complex series of biological events that lead to platelet activation and enhanced procoagulant activity of platelets.

We hypothesized that formation of coated platelets in dogs could be identified via externalization of platelet fibrinogen by use of an antibody-based flow cytometric assay, as has been described in humans. Furthermore, we hypothesized that recombinant human coagulation factor VIIa would bind preferentially to the population of coated platelets in dogs. The purpose of the study reported here was to assess by use of a flow cytometric assay the fibrinogen externalization from platelet \( \alpha \)-granules and compare it with P-selectin expression for conditions that involved the use of platelet-activating stimuli similar to those expected to be relevant at the site of a vascular injury. We further modified the assay so that we could assess the mechanism of action for recombinant human coagulation factor VIIa and determine whether it is comparable in humans and dogs.

**Materials and Methods**

**Sample**—Platelets were obtained from 4 dogs with hemophilia A, 4 dogs with hemophilia B, 4 dogs with von Willebrand disease, and 6 clinically normal mixed-breed dogs. All dogs were between 6 months and 10 years of age and were from the colony at the University of North Carolina at Chapel Hill. The dogs with hemophilia A had no detectable coagulation factor VII activity or antigen, and the resulting severe hemophilia was characterized by hemorrhage into the joints and soft tissues. Similarly, dogs with hemophilia B completely lacked coagulation factor IX activity and antigen in plasma and were also characterized by a severe hemophilic phenotype with hemorrhage into the joints and soft tissues. The dogs with von Willebrand disease were affected by a disease similar to type 3 von Willebrand disease in humans, which was characterized by undetectable von Willebrand factor antigen, multimers, or activity and a severe bleeding phenotype, especially at mucocutaneous sites. The clinically normal control dogs were hemostatically normal on the basis that results for a standard coagulation profile that included measurements of prothrombin time, activated partial thromboplastin time, fibrinogen concentration, and D-dimer concentrations were all within the respective laboratory reference ranges. Pooled plasma from the clinically normal dogs was used to define a factor concentration of 100% for coagulation factor VIII, coagulation factor IX, and von Willebrand factor. The study protocol was approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill.

**Sample collection**—Blood samples were collected from each dog 2 (dogs with hemophilia A, hemophilia B, and von Willebrand disease) or 3 (clinically normal dogs) times. Samples were collected from 4 to 8 dogs/d during a 14-day period; there was at least a 2-day interval between subsequent sample collections for each dog. During the period of sample collection, all dogs had no clinical signs of disease. Dogs were not given any medication for at least 14 days before the study or during the 14-day collection period.

Minimal manual restraint of dogs was used during sample collection. Samples of whole blood were collected via gentle manual occlusion of a cephalic vein and venipuncture with a 21-gauge butterfly needle. To minimize platelet activation artifacts, 1.5 mL of blood was aspirated through the butterfly needle and discarded before each 4.5-mL blood sample was collected into a tube that contained citrate (final concentration, 3.8%). Adequate anticoagulation was ensured by gentle inversion of the tube 5 times immediately after sample collection to mix the blood and citrate. To ensure that blood cellular components were within laboratory ref-
Preparation of washed platelets—Platelet-rich plasma was prepared from whole blood via centrifugation in polypropylene tubes at 275 × g for 5.5 minutes at 24°C. The platelet-rich plasma was acidified with 10% (vol/vol) acid-citrate-dextrose (2.5% [wt/vol] trisodium citrate, 1.5% [wt/vol] citric acid, and 2% [wt/vol] d-glucose); platelets were then centrifuged (300 × g for 10 minutes at 24°C). Platelets were then resuspended in HEPES-Tyrode buffer (15mM HEPES, 138mM NaCl, 5mM CaCl2, 2.7mM KCl, 1mM MgCl2, and 5.5mM d-glucose [pH, 6.3]) supplemented with 0.1% (wt/vol) bovine serum albumin and 10 µg of prostaglandin E1/µL. Platelets were pelleted again via centrifugation (300 × g for 10 minutes at 24°C) and then were resuspended in a small volume of reaction buffer (HEPES-Tyrode buffer, 5mM CaCl2, and 0.1% [wt/vol] bovine serum albumin [pH, 7.4]).

Flow cytometric assay—The flow cytometric assay was performed at the University of North Carolina core facility. Antibodies against CD61, P-selectin, and fibrinogen were obtained from commercial suppliers. Unlabeled anti–P-selectin and anti–fibrinogen antibodies were conjugated to a fluorochrome by use of commercially available conjugation kits used in accordance with the manufacturer’s instructions. Isotype control samples were prepared similarly, if they were not already conjugated to a fluorochrome. Antibody against P-selectin was purified by use of standard protein A affinity chromatography to remove gelatin from the formulation before conjugation to PerCP. Control experiments included use of FITC-labeled annexin-V (1:1,000 dilution) for assessment of phosphatidylserine externalization.

A 3-color platelet flow cytometric assay that identified platelets on the basis of constitutive membrane CD61 expression (first color) was configured to detect platelet activation by use of antibodies on the basis of the P-selectin expression (second color) and the coated platelet phenotype on the basis of fibrinogen externalization (third color). Specifically, samples (100 µL) that contained 4 × 10^5 washed platelets with no agonist; convulxin alone (optimized final concentration, 100 ng/mL); canine thrombin alone (optimized final concentration, 0.5 U/mL); or a combination of thrombin (optimized final concentration, 0.5 U/mL) and convulxin (optimized final concentration, 100 ng/mL) as well as anti–CD61-PE (optimized final concentration, 0.3 µg/µL), anti–CD62P-PerCP (optimized final concentration, 2.3 µg/µL), and anti–fibrinogen-APC (optimized final concentration, 0.4 µg/µL) or isotype-, concentration-, and fluorochrome-matched antibodies in reaction buffer were incubated for 10 minutes in the dark at 37°C with gentle rocking. Convulxin was used as a glycoprotein VI agonist substitute for collagen. The number of units of canine thrombin was determined by the manufacturer against the World Health Organization human standard thrombin (lot K) in a proteolytic assay with human fibrinogen as the substrate. In our hands, the amidolytic activity of 1 U of canine thrombin was approximately 180% the activity of 1 U of human standard thrombin when tested against the World Health Organization human standard thrombin in an assay that measured cleavage of the chromogenic substrate S-2238. Recombinant human coagulation factor VIIa was labeled with FITC at the active site as described elsewhere; FITC-rhFVIIa (0 to 10µM) was added in experiments designed to measure binding of recombinant human coagulation factor VIIa. Following incubation, 200 µL of 2% (vol/vol) paraformaldehyde in ice-cold HEPES-buffered saline solution (20mM HEPES and 150mM saline [0.9% NaCl] solution [pH, 7.4]) containing 3mM CaCl2 was added to each sample. After incubation (fixation) at 24°C for 20 to 60 minutes, samples were placed in polystyrene tubes and diluted in 1.25 mL of the HEPES-buffered saline solution containing CaCl2. Finally, the platelets were pelleted via centrifugation (1,500 × g for 10 minutes at 24°C) and resuspended in 0.5 mL of the HEPES-buffered saline solution containing CaCl2.

Samples were analyzed by use of flow cytometric analysis. Platelets were identified on the basis of their light-scattering properties and CD61 expression; platelet activation was measured on the basis of CD62P expression on the platelet surface, and formation of coated platelets was measured on the basis of fibrinogen externalization. Light scatter and fluorescence channels were set logarithmically. Voltage of the photomultiplier tube was set such that unstained platelets that had been stimulated with a thrombin-convulxin combination would be in the first decade, with a median fluorescence of approximately 2 arbitrary FUs for each fluorescence channel. All events were recorded until 10,000 events with platelet-like light-scatter properties were collected. On the basis of fluorescence of dual-agonist-stimulated isotype control samples and unstimulated control samples, threshold values were set at 20 FUs for CD61-PE, 30 FUs for CD62P-PerCP, 20 to 100 FUs for intermediate fibrinogen-APC, and > 100 FUs for high fibrinogen-APC. After initial optimization, settings were maintained throughout the study. For compensation of overlapping fluorescence, all experiments included unstained and single color–stained control samples consisting of platelets activated by stimulation with a thrombin-convulxin combination. To allow optimal compensation, unstained platelets were added to single color–control samples immediately before flow cytometric analysis. Data were automatically compensated and analyzed. For analysis, platelets were defined on the basis of gating on light-scatter properties plus CD61 expression (CD61-PE fluorescence). Platelets were then analyzed for P-selectin expression (CD62P-PerCP fluorescence) and fibrinogen externalization (fibrinogen-APC fluorescence).

In binding experiments, FITC-rhFVIIa was used to quantify the amount of platelet-bound recombinant human coagulation factor VIIa. Conversion of fluorescence to the amount of FITC-rhFVIIa per platelet was obtained by use of fluorescent calibra-
tion particles used in accordance with the manufacturer’s instructions. The binding equilibrium constant for the binding of recombinant human coagulation factor VIIa to canine platelets was calculated as described elsewhere.

Statistical analysis—Data were reported as mean ± SD. Values of P < 0.05 were considered significant. Normality of the complete data set was verified by use of D’Agostino and Pearson omnibus testing before comparison of mean data and data subsets. Values for agonist-specific P-selectin expression were compared by use of a paired t test (pairing on dog). A 1-way ANOVA was used to test the effect of hemostatic phenotype on generation of coated platelets; a Bonferroni multiple comparison test was used to make comparisons among all phenotypes. A 2-way ANOVA was used to test effects of interindividual variation (effect of dog) and day-to-day variation (effect of day of experiment) on the assay.

Results
Platelet preparation, CD61 expression, and basal P-selectin expression—Platelets were isolated from blood samples from dogs with hemophilia A, hemophilia B, and von Willebrand disease and hemostatically normal dogs. Results for CBCs were within laboratory reference ranges for all samples (data not shown). Similar to data for humans, CD61 expression was increased over baseline values after stimulation with a combination of thrombin and convulxin (Figure 1). Of the platelets, 1% to 2% had marginally positive results for P-selectin without agonist stimulation (Table 1).

P-selectin expression in canine platelets in response to thrombin and convulxin—The P-selectin expression in canine platelets was investigated for stimulus conditions with various agonists (no agonist, convulxin alone, thrombin alone, and thrombin and convulxin in combination; Table 1). When data from all 18 dogs were pooled, we found that P-selectin expression in canine platelets was most pronounced when platelets were stimulated with the thrombin-convulxin combination (mean ± SD proportion of platelets with positive results for P-selectin, 93 ± 2%). Stimulation of the platelets with a single agonist resulted in a significantly (P < 0.001) lower proportion of platelets with positive results for P-selectin (mean ± SD of 60 ± 9% for thrombin and 55 ± 2% for convulxin), compared with the results after stimulation with the thrombin-convulxin combination; there also was a significant (P = 0.039) difference between the percentages of platelets positive for P-selectin after stimulation with thrombin alone or convulxin alone. Stimulation with the thrombin-convulxin combination revealed that only a single population of platelets positive for P-selectin was identifiable and that differentiation between coated and noncoated platelets was not possible.

Table 1—Mean ± SD percentage of canine platelets with positive results for P-selectin expression after the platelets were stimulated with agonists.

<table>
<thead>
<tr>
<th>Hemostatic phenotype</th>
<th>No. of dogs</th>
<th>No agonist</th>
<th>Thrombin (0.5 U/mL)</th>
<th>Convulxin (100 ng/mL)</th>
<th>Thrombin and convulxin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinically normal</td>
<td>6</td>
<td>0.8 ± 0.6</td>
<td>56.7 ± 6.4</td>
<td>56.0 ± 11.9</td>
<td>92.3 ± 3.2</td>
</tr>
<tr>
<td>Hemophilia A</td>
<td>4</td>
<td>1.8 ± 1.0</td>
<td>64.3 ± 7.9</td>
<td>62.0 ± 6.3</td>
<td>94.0 ± 1.4</td>
</tr>
<tr>
<td>Hemophilia B</td>
<td>4</td>
<td>2.2 ± 1.3</td>
<td>68.6 ± 6.5</td>
<td>54.5 ± 4.9</td>
<td>93.5 ± 0.9</td>
</tr>
<tr>
<td>von Willebrand disease</td>
<td>4</td>
<td>1.2 ± 0.7</td>
<td>53.5 ± 9.8</td>
<td>45.0 ± 13.8</td>
<td>92.6 ± 1.7</td>
</tr>
</tbody>
</table>

Percentages represent percentage of P-selectin–positive platelets in response to the indicated agonists for platelets obtained from the indicated number of dogs (2 dogs with hemophilia A, hemophilia B, and von Willebrand disease) or 3 (clinically normal control dogs) separate experiments. For example, a mean ± SD of 0.8 ± 0.6% of the platelets from the 6 clinically normal dogs was positive for P-selectin when no agonist was added during incubation and the experiment was repeated 3 times. Expression of P-selectin was detected by use of a monoclonal anti-CD62P antibody.
Fibrinogen externalization as a marker of coated platelets—Fibrinogen externalization from α-granules was used to identify coated platelets in dogs. The coated platelet phenotype was attained when the thrombin-convulxin combination was used but not when either agonist was used alone (Figure 2; Table 2). Fibrinogen externalization above the threshold value had a bimodal pattern and could be classified as intermediate or high (Figure 1). This facilitated differentiation between coated and noncoated canine platelets; this differentiation was not possible via monitoring of P-selectin expression. Analysis of the data revealed no evidence that platelets could externalize fibrinogen without also expressing P-selectin. In contrast, evidence was obtained that canine platelets were able to express P-selectin without concurrent fibrinogen externalization. Control experiments conducted with FITC-labeled annexin-V verified that similar to the results for human platelets, fibrinogen externalization was colocalized to platelets with high phosphatidylserine externalization (data not shown).

Coated platelets from dogs with hemophilia A, hemophilia B, and von Willebrand disease and clinically normal dogs—To assess whether the potential for generation of coated platelets differed markedly among dogs with congenital bleeding disorders, results for dogs with 3 such conditions were compared with results for hemostatically normal dogs. The flow cytometric assay was performed with platelets from dogs with various hemostatic phenotypes (4 dogs with hemophilia A, 4 dogs with hemophilia B, 4 dogs with von Willebrand disease, and 6 clinically normal dogs). The potential for generating coated platelets did not differ significantly (P = 0.103) in dogs with these hemostatic phenotypes (Table 2). Interestingly, 1 dog with von Willebrand disease had a noticeably lower response to convulxin (mean, 28% of platelets were positive for P-selectin), compared with results for the other 3 dogs with von Willebrand disease (mean, 41%, 54%, and 58% of platelets were positive for P-selectin, respectively); these results differed significantly (P = 0.046; 1-sample t test for the mean of the 1 dog with von Willebrand disease vs the mean of the other 3 dogs with von Willebrand disease). There was no further exploration of the possible cause for this finding.

Variability in P-selectin expression and fibrinogen externalization—To assess the variability of P-selectin expression and fibrinogen externalization as markers of platelet activation and to identify possible sources of the variation, the flow cytometric assay was performed in duplicate with platelets that were obtained from all 6 clinically normal dogs 3 separate times and stimulated with the thrombin-convulxin combination. There was significant variation among dogs for P-selectin expression (P = 0.003) and fibrinogen externalization (P < 0.001). Thus, both markers had dog-dependent results for platelets stimulated with the thrombin-convulxin combination. There was significant variation among dogs for fibrinogen externalization (P < 0.001) but not for maximal P-selectin expression (P = 0.18). For P-selectin expression,
48% of total variance was attributable to a dog-dependent effect, and 6% of the variance was attributable to the day of sample collection. For fibrinogen externalization, these values were 51% and 24%, respectively.

Binding of human recombinant coagulation factor VIIa to canine platelets—The FITC-rhFVIIa was used to evaluate in vitro binding of recombinant human coagulation factor VIIa to canine platelets. In 5 experiments conducted with platelets obtained from the 4 dogs with hemophilia A, saturable binding (mean ± SD binding equilibrium constant, 2.6 ± 0.5 µM) of FITC-rhFVIIa to coated platelets was identified (Figure 3). Specific binding was calculated as binding to coated platelets minus binding to unstimulated platelets. In contrast, noncoated and unstimulated platelets bound FITC-rhFVIIa in a nonspecific manner; fluorescence intensity was linearly correlated with the FITC-rhFVIIa concentration applied. Results of 2 preliminary experiments suggested similar binding to platelets (mean ± SD binding equilibrium constant, 2.4 ± 1.3 µM) as that obtained for the 6 clinically normal dogs. In the 5 experiments conducted with platelets obtained from the 4 dogs with hemophilia A, mean maximal binding capacity of coated platelets for FITC-rhFVIIa was 285,000 ± 49,000 molecules/coated platelet.

Discussion

Analysis of the results of the study reported here revealed that coated platelets in dogs can be identified with a 3-color antibody-based flow cytometric assay by use of CD61 as a platelet marker, P-selectin as a general marker of activation, and α-granule fibrinogen as a marker specific for coated platelets. To our knowledge, this is the first report in which endogenous platelet fibrinogen has been used to identify coated platelets in dogs. Differential α-granule release has been identified in human platelets,33 and results of the present study indicated that α-granule degranulation in canine platelets may also be a finely orchestrated process because distinct patterns were detected for expression of P-selectin and fibrinogen.

We used P-selectin as a general marker of platelet activation. This allowed us to estimate the degree of activation, even when there was a lack of formation of coated platelets. The P-selectin data in the present study are comparable to results in other studies of human1 and canine17,19 platelets. However, the study reported here had better resolution between no response and a fully positive response because only a few unstimulated platelets expressed P-selectin.

Figure 3—Binding of FITC-rhFVIIa to platelets in dogs. A—Binding of FITC-rhFVIIa to unstimulated platelets (left column) and platelets stimulated with a thrombin-convulxin combination (right column) after incubation without FITC-rhFVIIa (upper plot in each pair) and with 1,000 nM FITC-rhFVIIa (lower plot in each pair) was analyzed. Coated platelets formed after stimulation with the thrombin-convulxin combination (ie, right column) were identified as the population of platelets with high fibrinogen externalization in Figures 1 and 2. See Figure 1 for key. B—Specific binding of FITC–rhFVIIa to coated platelets (white triangles) was calculated as total binding to coated platelets (black squares) minus binding to unstimulated platelets (black circles). Notice the binding of FITC-rhFVIIa to platelets stimulated with a thrombin-convulxin combination that had low fibrinogen externalization (white circles). Symbols are shown with the corresponding regression lines where applicable. Data are results for a representative experiment that involved the use of platelets from a dog with hemophilia A.
55% to 60% of the platelets stimulated with thrombin or convulxin expressed P-selectin, and typically > 90% of platelets stimulated with the thrombin-convulxin combination expressed P-selectin.

Biotinylated human fibrinogen has been used to identify coated platelets in dogs.31 By use of this approach, investigators in another study17 found that approximately 40% of the platelets from healthy dogs had the coated platelet phenotype when platelets were stimulated with a combination of 0.5 U of bovine thrombin/mL and 100 ng of convulxin/mL. In the present study, approximately 70% of platelets from clinically normal dogs had the coated platelet phenotype. This disparity has also been found for human platelets20,21,34 and could probably be attributed to slight differences in experimental conditions.

To evaluate the potential for the formation of coated platelets in dogs with various hemostatic phenotypes, platelets from clinically normal dogs and dogs with congenital defects of primary and secondary hemostasis (ie, von Willebrand disease, hemophilia A, and hemophilia B) were tested. Analysis of the data supported that the ability of dogs to generate coated platelets was independent of the bleeding phenotypes for hemophilia A, hemophilia B, and von Willebrand disease.

Binding experiments with FITC-rhFVIIai revealed that recombinant human coagulation factor VIIa bound preferentially to coated platelets in dogs. These results suggest that exposed subendothelial collagen and initially formed thrombin can induce formation of coated platelets in vivo and can thereby localize pharmacologically administered recombinant human coagulation factor VIIa to sites of vessel wall injury.35 Although it is possible that labeling of the active site of recombinant human coagulation factor VIIa marginally increases the affinity for platelets (in our experience, coated as well as noncoated platelets), FITC-rhFVIIai was used in the present study because it allowed direct comparison of data on binding of FITC-rhFVIIai to canine platelets with similar data published on human platelets. The affinity of human FITC-rhFVIIai for canine platelets (mean ± SD binding equilibrium constant, 2.6 ± 0.5µM) was found to be in the same range as that reported in a study35 of human coated platelets, which bound FITC-rhFVIIai with a mean binding equilibrium constant of 1.2 ± 0.2µM and had a maximal capacity of approximately 200 × 10^3 FITC-rhFVIIai molecules/platelet. This suggests similar mechanisms of action for recombinant human coagulation factor VIIa in dogs and humans. In addition, recombinant human coagulation factor VIIa can be efficacious for correcting the bleeding phenotype in dogs with hemophilia A, hemophilia B, or coagulation factor VII deficiency.31,34 However, recombinant human coagulation factor VIIa is immunogenic in dogs. Therefore, although anti–recombinant human coagulation factor VIIa antibodies do not apparently cross-react with canine coagulation factor VII or coagulation factor VIIa and aggravate the preexisting bleeding phenotype of hemophilia A, hemophilia B, or coagulation factor VII deficiency in dogs or cause any other signs of morbidity (or even death), studies of longer duration are needed to elucidate the potential consequences of the use of recombinant human coagulation factor VIIa in dogs.

Given the good resolution for P-selectin obtained in the present study, it should be possible to use the flow cytometric assay to investigate general platelet activation in the pathophysiologic processes of disease states (eg, thromboembolism) in dogs with diseases (eg, immune-mediated hemolytic anemia) that predispose to this often fatal complication. In dogs with immune-mediated hemolytic anemia, platelets circulate in an activated state,36 and thromboembolism is a major cause of morbidity and death.37–40 However, the reason that platelets from dogs with primary acquired immune-mediated hemolytic anemia are often hyperresponsive is currently not known. This hyperresponsiveness could be evaluated further by also assessing the potential of platelets from dogs with immune-mediated hemolytic anemia to have the coated platelet phenotype.

We concluded that the antibody-based flow cytometric assay for detection of coated platelets in dogs that was developed in the study reported here has the potential to become a valuable tool to facilitate better understanding of the role of coated platelets in hemostatic and thrombotic disorders in dogs and the treatment of dogs with such disorders. In addition, the data reported here support the hypothesis that formation of coated platelets in dogs and humans is via a similar mechanism; thus, the potential for the use of dogs in the study of bleeding disorders in humans is further strengthened. Flow cytometric data reported here revealed localization of recombinant human coagulation factor VIIa to coated activated platelets in dogs. Thus, the findings support a similar mechanism of action for recombinant human coagulation factor VIIa in both humans and dogs.

a. ABX Micros ABC Vet, HORIBA ABX, Irvine, Calif.

b. Sigma-Aldrich, St Louis, Mo.
c. PE-conjugated monoclonal mouse anti-human CD61, clone V2Y/91, Santa Cruz Biotech, Santa Cruz, Calif.
d. Monoclonal mouse anti-human CD61, clone 9E13, Santa Cruz Biotech, Santa Cruz, Calif.
e. Monoclonal mouse anti-human CD62P, clone 1E3, Santa Cruz Biotech, Santa Cruz, Calif.
h. Normal mouse IgG2a, X0903, Dako, Glostrup, Denmark.
i. Rabbit normal IgG, X0903, Dako, Glostrup, Denmark.
j. PE-conjugated normal mouse IgG1, Santa Cruz Biotech, Santa Cruz, Calif.
k. Annexin V-FITC, 556419, BD Pharmingen, Franklin Lakes, NJ.
l. Polyclonal rabbit anti-human fibrinogen, A0080, Dako, Glostrup, Denmark.

ajvr:a110494
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