Immune-mediated hemolytic anemia is a well-characterized systemic inflammatory disorder of dogs that can lead to fatal thromboembolism. Large and small vessel thrombi are commonly identified in the spleen, lungs, and catheterized veins of dogs with IMHA that die or are euthanatized. Although the pathogenesis of thrombosis is not fully defined in this disorder, an increase in platelet reactivity secondary to inflammation has been proposed as a contributing factor. Consistent with the belief that heightened platelet reactivity associated with systemic or vascular inflammation may be involved with thromboembolism in dogs with IMHA, improved survival was suggested in a retrospective study of dogs with IMHA treated with ULDAsp (0.5 mg/kg, PO, q 24 h) combined with glucocorticoids and azathioprine, compared with dogs treated with glucocorticoids and azathioprine or glucocorticoids, azathioprine, and mixed-molecular-weight heparin.1

Influence of treatment with ultralow-dose aspirin on platelet aggregation as measured by whole blood impedance aggregometry and platelet P-selectin expression in clinically normal dogs

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**Objective**—To evaluate the influence of treatment with ultralow-dose aspirin (ULDAsp) on platelet aggregation, P-selectin (CD62P) expression, and formation of platelet-leukocyte aggregates in clinically normal dogs.

**Animals**—18 clinically normal dogs.

**Procedures**—Studies were conducted before and 24 hours after ULDAsp administration (0.5 mg/kg, PO, q 24 h, for 2 days). Whole blood impedance aggregometry for the assessment of platelet function was performed with sodium citrate-anticoagulated blood and aggregation agonists (ADP at 20, 10, and 5 µmol/L, collagen at 10, 5, and 2 µg/mL). Onset, maximum response, and rate of platelet aggregation were recorded. Flow cytometric assays were configured to detect thrombin-induced CD62P expression and platelet-leukocyte aggregates in EDTA-anticoagulated whole blood. Externalized platelet CD62P and constitutive CD61 (GPIIIa) were labeled with antibodies conjugated to phycocerythrin (PE) and fluorescein isothiocyanate (FITC), respectively. Red blood cell-lysed paraformaldehyde-fixed EDTA-anticoagulated whole blood was dual labeled with CD61-FITC and a panleukocyte antibody (CD18-FITC) to characterize platelet-leukocyte aggregates.

**Results**—ULDAsp significantly delayed platelet aggregation onset with ADP at 20 µmol/L by 54% to 104%, attenuated maximum aggregation with various concentrations of ADP and collagen by ≥ 41%, and slowed aggregation rate with the highest ADP and collagen concentrations by ≥ 39%. Depending on the parameter tested, up to 30% of dogs failed to have an ULDAsp effect. Thrombin stimulation significantly increased CD62P expression in platelets and platelet-leukocyte aggregates, but ULDAsp did not alter basal or thrombin-stimulated CD62P expression.

In humans, low-dose aspirin administration is only 1 of many antithrombotic approaches used to decrease morbidity and death associated with thromboembolic disorders. In fact, treatment regimens for thromboembolic events are diverse and undergoing intense scrutiny in human medicine. Recent experimental work suggests that shear gradient–dependent platelet aggregation may importantly contribute to thrombus initiation in some diseases. How- ever, current prophylactic and interventional treatments in humans typically involve mixed- or low–molecular-weight heparin, coumadin, fondaparinux, or combinations of these treatments for deep vein thrombosis (associated with low shear), and aspirin, dipyriramole, ticlopidine, or clopido- grel for arterial thrombosis or atherosclerosis (associated with high shear).

Studies on dogs with IMHA suggest that plate- let hyperactivity and systemic inflammation may be associated with thromboembolic complications. Seemingly, if thrombi in dogs with IMHA predominate in the low shear venous vasculature where platelet aggregation may play a minor role in thrombus formation, the utility of aspirin as an antithrombotic agent has been questioned. Aspirin irreversibly acetylates a specific serine residue in cyclooxygenase, inhibiting thrombox- ane synthesis in platelets. Thromboxane promotes platelet activation and platelet aggregation by signaling through a surface membrane G-protein–coupled receptor. However, there is wide variability in platelet response to thromboxane owing to dysfunc- tion of surface G-protein signaling in as many as 70% of dogs. These collective findings raise doubts about the potential benefits of aspirin as an antiaggregatory agent in dogs. Nevertheless, the antiprostaglandin effects of aspirin may attenuate prothrombotic inflammation in dogs with IMHA.

Laboratory markers to detect platelet activation include platelet membrane expression of P-selectin and fibrinogen. Selectin, an α-granule protein externalized in the process of platelet degranulation, functions as an adhesion molecule mediating platelet binding to leukocytes and endothelial cells. However, once exposed, membrane-surface P-selectin can undergo internalization, limiting its reliability as a sus- tained marker of platelet activation.

Nevertheless, a study in dogs with IMHA demonstrated an approximately 8-fold increase in P-selectin expression on circulating platelets, compared with that of healthy dogs, and 2-fold greater P-selectin expression following in vitro activation of platelets from dogs with IMHA. Apparently, many dogs with IMHA have platelets that circu- late in an activated state and have an exaggerated in vitro agonist response.

Because detection of surface markers of platelet activation may not correlate directly with functional platelet responses, several methods have been devised to assess platelet function. The in vitro mucosal bleeding time is a screening test of primary hemostasis used to detect failure of hemostatic plug formation. The test is subject to procedural variations, including the depth and width of the bleeding time incision, animal excitement and blood pressure, and mechanical vari- ables (eg, physical dislodgement of the primary clot and interoperator differences). Furthermore, this test is nonspecific as a test of platelet function because it is af- fected by blood viscosity (anemia), hemostatic proteins (eg, von Willebrand factor and fibrinogen), and platelet number. An alternate in vitro method of assessing platelet function is to measure closure time by use of a platelet function analyzer, currently popular as a point-of-care platelet assessment tool. This rapid assay measures platelet adhesion and platelet aggregation in whole blood aspirated under high shear through a small aperture coated with collagen and either epinephrine or ADP. Closure time as determined on a platelet function analyzer, however, is influenced by some of the same nonspecific factors that affect the buccal mucosal bleeding time, including plasma von Willebrand factor concentration, platelet count, and blood viscosity.

If low shear stress–associated thrombus formation indeed characterizes pathological clot formation in dogs with IMHA, this testing method may be inappropriate. Whole blood impedance aggregometry represents a more specific screening method to as- sess platelet function on the basis of platelet response to stimulation with a variety of in vitro agonists (eg, epinephrine, collagen, and ADP). Furthermore, whole blood impedance aggregometry does not challenge platelet response under shear-stress conditions.

The study reported here was undertaken to explore the effect of in vivo treatment with ULDAsp on platelets in clinically normal dogs. Our goals were to examine the influence of pretest variables (ie, different anticoag- ulants, time delay to analysis, and varied concentrations of in vitro agonists) and ULDAsp treatment on platelet aggregation as measured by whole blood impedance aggregometry and the impact of ULDAsp on basal and thrombin-stimulated platelet P-selectin expression and platelet-leukocyte aggregate formation as determined by flow cytometry. This work was undertaken to estab- lish methodologies and reference intervals for future investigation of platelet reactivity in dogs with IMHA.

Materials and Methods

Animals—All dogs were privately owned pets in good health. Informed use and consent compliant with the Institutional Animal Care and Use Guidelines of Cornell University were followed. Dogs are described in detail under study sections.

Study 1—As a preliminary study, blood collected from 4 adult clinically normal dogs (median age, 5.5 years [range, 4 to 10 years]; median body weight, 27.5 kg [range, 22 to 33 kg]; 2 neutered females, 1 neutered male, and 1 sexually intact male; 2 Australian Cattle Dogs and 2 Golden Retrievers) was used to investigate whether anticoagulation with CPD (0.016M citric acid, 0.09M sodium citrate dihydrate, 0.016M monomeric sodium phosphate monohydrate, and 0.14M dextrose monohydrate; pH adjusted to 7.4 with NaOH; 1:10), ACD (4 mM citric acid, 0.08M sodium citrate dihy- drate, and 0.14M dextrose monohydrate; 1:10), or 3.2% (0.109M) sodium citrate was preferable for in vi-
tro platelet aggregation assessments. Duplicate trials of CPD and sodium citrate were done in 2 dogs to determine which anticoagulant provided best performance (ie, longevity of blood anticoagulation and consistency of platelet aggregation results). Polypropylene syringes were used to collect blood from a cephalic or lateral saphenous vein; blood was promptly and gently mixed with the anticoagulant, placed in a 37°C preheated tube, and transported to the laboratory where it was maintained at 37°C. All blood samples were collected from dogs in the early morning in a calm environment, and all dogs had platelet counts > 250,000/µL.

Study 2—Platelet aggregation responses before and after ULDAsp administration were studied in 18 adult clinically normal dogs (median age, 6 years [range, 3 to 13 years]; median body weight, 30.5 kg [range, 5.1 to 39.4 kg]; 12 neutered females, 5 neutered males, and 1 sexually intact male; 8 mixed-breed large dogs, 2 Australian Cattle Dogs, and 1 each of Jack Russell Terrier, Rat Terrier, Golden Retriever, German Shepherd Dog, American Staffordshire Terrier, Greyhound, Rottweiler, and Beagle). Aspirin was administered PO at 0.5 mg/kg every 24 hours for 2 days, then blood was collected 24 hours after the second dose. Platelet aggregation responses were evaluated for 9 dogs before ULDAsp administration, then reassessed after ULDAsp administration. Platelet aggregation responses also were evaluated for 9 dogs with ULDAsp treatment first, followed by reasessments after a 10-day washout interval. Blood samples were collected as outlined in study 1, except collection syringes were primed with 3.2% sodium citrate. All dogs had platelet counts > 250,000/µL. Blood samples from 10 of these dogs were tested at 1-hour intervals for 8 hours after blood collection to determine the influence of delayed analysis on platelet aggregation responses.

Whole blood impedance aggregometry for studies 1 and 2—The impedance aggregometer was turned on for 30 minutes on each skin use. Modified Tyrode's buffer (140 mM NaCl, 4.5 mM MgCl2, 2.6 mM KCl, 5.5 mM glucose, 11.9 mM NaHCO3, 0.5 mM Na2HPO4, 2 mM CaCl2, and 2 U heparin/mL; pH, 7.4) was maintained at 37°C in a water bath. Platelet aggregation responses were initiated by combining 450 µL of Tyrode's buffer with 450 µL of blood in aggregometer cuvettes containing a stirring magnet. The aggregation electrode was gently cleansed with purified, deionized water and wiped dry before each platelet aggregation recording; a clean cuvette was used for each analysis. By use of a chart recorder, baseline and calibrations were established. Blood and Tyrode's buffer were mixed for 2 minutes before addition of activating agonists.

For study 1, the effect of ADP on platelet aggregation was evaluated at a concentration of 20 µmol of ADP/L for each of the 4 dogs. For study 2, the effects of ADP (at 20, 10, and 5 µmol/L) and collagen (ie, native collagen fibrils [type I] from equine tendons suspended in isotonic glucose solution; pH, 2.7; at 10, 5, and 2 µg/mL) on platelet aggregation was evaluated. Platelet aggregation was tested with at least 1 concentration of ADP (ie, 20 µmol/L) and all concentrations of collagen for all 18 dogs in study 2; effects of all concentrations of ADP (ie, 20, 10, and 5 µmol/L) on platelet aggregation were evaluated for 10 dogs in study 2. In blood samples from each dog, the highest concentration of agonist was tested first. Onset (seconds), maximum response (Ω), and rate (Ω/s) of platelet aggregation were determined for each sample. For the 10 dogs in study 2 that had blood samples analyzed at 1-hour intervals for 8 hours, the evaluated agonist was ADP at 20 µmol/L.

Study 3—A subset of dogs (n = 10) from study 2 were used to investigate P-selectin expression and platelet-leukocyte aggregate formation before and after ULDAsp treatment. A whole blood flow cytometric assay previously configured to detect platelet activation on the basis of thrombin-induced platelet P-selectin externalization and platelet-derived microparticle release was adapted for this study. Activation reactions were performed in 5-mL polystyrene tubes in a 100-µL total assay volume. The reaction mixture contained 6 µL of fresh EDTA-anticoagulated whole blood, 90 µL of PBS solution with bovine serum albumin, and 0.4 mM gly-pro-arg-pro-NH2. Platelets were stimulated with thrombin (final concentration, 2 U/mL) for 10 minutes at 37°C, then fixed for 30 minutes by the addition of a one-tenth volume of 4% paraformaldehyde buffer. After fixation, fluorescent-conjugated antibodies were added directly to the reaction tubes to identify platelets on the basis of constitutive membrane GPIIIa (CD61-FITC [clone S221]; 0.025 ng/100 µL) and to detect membrane expression of P-selectin (CD62P-PE [clone AC1.2]; 8 µL/100 µL). These commercially available antibodies have been used in previous cytometry studies to identify canine platelet antigens. One set of the nonstimulated control reaction tubes was also double-labeled with CD61-FITC and a PE-isotype control (mouse IgG1, PE). Sample tubes were labeled in the dark for 30 minutes at room temperature (approx 25°C), followed by quench-dilution with 1 mL of reaction buffer, then analyzed within 60 minutes. Stimulated and nonstimulated control reactions were performed in triplicate.

The leukocyte fraction was prepared to calibrate cytometer gates for identification of granulocytes, monocytes, and lymphocytes. A paraformaldehyde buffer-fixed EDTA-anticoagulated whole blood sample (1.8 mL blood with 200 µL paraformaldehyde buffer) was incubated for 30 minutes at room temperature (approx 25°C), followed by quench-dilution with 1 mL of reaction buffer, then analyzed within 60 minutes. Stimulated and nonstimulated control reactions were performed in triplicate.
ies were initially used to validate gating based solely on forward scatter and side scatter characteristics of the cell suspensions (data not shown). Thereafter, platelet-leukocyte aggregates were assessed by use of dual antibodies to achieve panleukocyte-platelet localization.38 The influence of platelet activation on platelet-leukocyte interaction was determined by evaluating leukocyte preparations after in vitro thrombin activation.

Flow cytometry for study 3—Samples were analyzed by use of a cytometer1 and software program2 using logarithmic gain setting for forward and side scatter characteristics. After initial optimization and compensation, the same settings were used throughout the study. The flow cytometer calibrations were checked daily with specific calibration beads.3 Fluorescence compensation was determined by use of single-fluorescence labeled cells. Whole blood samples were gated to collect 10,000 CD61 positive events (ie, platelets or platelet-derived microparticles). For analysis, platelets and platelet-derived microparticles were first discriminated on the basis of their CD61-FITC fluorescence, then all CD61-positive events were subjected to 2-color analysis to identify the proportion of platelets binding CD62P-PE (denoting P-selectin expression; Figure 1). The y-axis analysis threshold for CD62P-PE positive events was set on the basis of the fluorescence of the isotype-PE labeled platelets. Platelet-derived (CD61-positive) membrane microparticles and aggregates were identified on the basis of forward scatter properties with the y-axis threshold set at $10^4$ forward scatter to represent the lower boundary of forward scatter for nonstimulated platelets. To identify leukocyte distributions, WBCs in RBC-lysed whole blood were labeled with CD18-PE and CD3-FITC and independently with B-cell-PE antibodies. Thereafter, 2-color analysis (CD18-PE and CD61-FITC; Figure 2) was used to determine platelet binding with leukocytes from dogs before and after ULDAasp treatment. Two-color analysis (CD62P-PE with CD61-FITC) was used to identify platelet-leukocyte aggregates expressing P-selectin after in vitro thrombin platelet activation.

Statistical analysis—Data were examined for Gaussian distribution by use of box and whisker plots

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Figure 1—Gating logic for identification of platelet aggregates, platelets, and platelet microparticles in EDTA-anticoagulated whole blood. A—Regions identifying platelet distributions. B—Histograms demonstrating CD62P-PE positive platelets (M2 region) in nonstimulated and thrombin-stimulated blood. C—Dot plots demonstrating platelet events with (upper right quadrant) and without (lower right quadrant) P-selectin expression in nonstimulated and thrombin-stimulated blood. FSC = Forward scatter. M1 = CD62P-PE negative platelets. M2 = CD62P-PE positive platelets.
and the Kolmogorov-Smirnov test. Platelet aggregation variables at timed intervals for CPD, ACD, and sodium citrate–anticoagulated blood stimulated with ADP (20 µmol/L) were not normally distributed and were expressed as median and range. Onset and maximum response of platelet aggregation between the eight 1-hour intervals for sodium citrate–anticoagulated blood and between anticoagulants at 1, 3, 5, 7, 9, and 12-hour intervals were examined by use of the Wilcoxon signed rank test with Bonferroni correction (P = 0.006 for the eight 1-hour intervals and P = 0.008 between anticoagulants). The coefficient of variation percentage was calculated for the 1-hour intervals of the sodium citrate–anticoagulated samples for each dog. Platelet aggregation variables for sodium citrate-anticoagulated blood before and after ULDAsp treatment were normally distributed and expressed as mean ± SD. Differences between anticoagulant groups were determined by use of an ANOVA for repeated measures; a value of P < 0.05 was considered significant.

Expression of P-selectin was determined by calculating the percentage of platelets labeled with CD61 that were concurrently labeled with CD62P. The percentage of CD62P positive cells following in vitro thrombin activation were compared with the percentage of CD62P positive cells in nonstimulated samples. Platelet-leukocyte aggregates (percentage of gated cells) were determined with dual antibodies (CD61-FITC with CD62P-PE or CD18-PE), and leukocyte distributions were compared with and without thrombin stimulation and from dogs before and after ULDAsp treatment. These data were not normally distributed and expressed as median and range. Differences were identified by use of the Wilcoxon signed rank test with a 2-sided value of P ≤ 0.05.

**Results**

ADP-stimulated platelet aggregation responses with CPD or ACD—In study 1, blood anticoagulated with CPD or ACD inconsistently allowed for ADP (20 µmol/L)-stimulated platelet aggregation testing. For blood samples anticoagulated with CPD, only those from 2 of the 4 dogs could be tested (1 through 24 hours and 1 through 4 hours) before clot formation occurred; blood samples from the other 2 dogs formed clots within 2 hours. Similarly, for blood samples anticoagulated with ACD, only those from 3 of the 4 dogs could be tested (1 through 18 hours, 6 through 36 hours, and 1 through 4 hours) before clot formation occurred; blood samples from the other 2 dogs formed clots within 2 hours. Similarly, for blood samples anticoagulated with CPD and ACD, platelet aggregation variables were not normally distributed and were expressed as median and range. Differences were identified by use of the Wilcoxon signed rank test with a 2-sided value of P ≤ 0.05.
In study 2, a significant (treatment, P < 0.001) increase in P-selectin expression and platelet-leukocyte aggregate formation before and after ULDAsp treatment—In study 3, in vitro thrombin stimulation induced a significant (P < 0.001) increase in P-selectin expression on platelets, platelet aggregates, and platelet-derived microparticles, compared with basal (nonstimulated) values (Table 1). However, treatment of dogs with ULDAsp had no significant effect on basal or thrombin-stimulated P-selectin expression. A significant (P = 0.009) increase in platelet-leukocyte aggregates expressing P-selectin (CD62P) followed thrombin stimulation, albeit cell counts were low (Table 2). A change in platelet-leukocyte aggregation based on CD61 detection was not apparent following thrombin stimulation or after ULDAsp treatment (Tables 2 and 3). Neither thrombin stimulation nor ULDAsp treatment altered leukocyte distribution (data not shown).

**Discussion**

To date, the influence of ULDAsp in dogs with IMHA treated concurrently with azathioprine and glucocorticoids has only been described in a single retrospective study. Although that study revealed improved survival of dogs with IMHA with the addition of...
ULDAsp to the treatment regimen, further exploration is warranted. Possible beneficial effects of aspirin could reflect inhibition of thromboxane-induced platelet activation, modulatory effects on vascular endothelium

Figure 4—Mean ± SD measurements (onset [A and B], maximum response [C and D], and rate [E and F]) as determined by whole blood impedance aggregometry for assessment of platelet aggregation with agonists ADP and collagen in 10 clinically normal dogs before (black bars) and after (white bars) ULDAsp treatment.

Table 1—Median (range) P-selectin expression (ie, percentage of gated cells) in platelet aggregates, platelets, and platelet microparticles before and after thrombin stimulation in blood collected from 10 clinically normal dogs before and after ULDAsp treatment.

Table 2—Median (range) platelet-leukocyte aggregates (ie, percentage of gated cells) before and after thrombin stimulation in blood collected from 10 clinically normal dogs before ULDAsp treatment.
and systemic inflammation, and aspirin-altered drug-protein binding. However, meaningful evaluation of the influence of ULDAsp on platelet reactivity in dogs with IMHA will require standardized protocols that can be conducted within a reasonable time frame by use of a relevant method of assessment that will allow prospective study.

As such, we investigated the usefulness of different anticoagulants (ie, CPD, ACD, and sodium citrate) in whole blood samples, the allowable time delay between blood collection and platelet aggregation assessments, and the influence of different agonist concentrations of ADP and collagen by use of impedance platelet aggregometry. We found that whole blood anticoagulated with 3.2% sodium citrate (1:9), as provided in commonly available blood collection tubes, worked well. Platelet aggregation was reliably assessed in blood within 8 hours of collection. However, it is important to recognize that we took careful measures to minimize iatrogenic platelet activation (ie, single needle phlebotomy from a fresh vein, dogs maintained in a calm environment, blood gently mixed with anticoagulant during collection, preheated polystyrene collection tubes, and 37°C constant incubation). Although there are many platelet activation agonists (eg, arachidonic acid, thromboxane, thrombin, ADP, collagen, epinephrine, and phorbol myristate acetate), we selected thrombin for flow cytometry and ADP and collagen for whole blood impedance aggregometry studies because these agonists are among the most commonly used that reliably activate platelets. We did not select arachidonic acid as an agonist because canine platelets respond weakly to both ADP and collagen in clinically normal dogs. In fact, following ULDAsp administration, attenuated platelet aggregation responses to ADP were documented for 14 of 18 dogs (20 µmol/L ADP) and 8 of 10 dogs (5 or 10 µmol/L of ADP) and to the higher concentrations of collagen for 16 of 18 dogs (5 µg/mL of collagen) and 14 of 18 dogs (10 µg/mL of collagen). In dogs treated with ULDAsp, the reduced maximum response for collagen-initiated platelet aggregation was expected, consistent with attenuation of the secondary platelet response. Studies in other species (including dogs) demonstrate that aspirin and other inhibitors of arachidonic acid metabolism can inhibit collagen-induced platelet aggregation.

Flow cytometry enables evaluation of single cell response to physiologic and pathological conditions. We used whole blood or cell suspension preparations in flow cytometric assays to differentiate cell types (ie, platelets, granulocytes, monocytes, and lymphocytes), detect platelet P-selectin expression, and identify platelet-leukocyte aggregates. This study demonstrated no difference in basal P-selectin expression or platelet-leukocyte aggregate formation following ULDAsp treatment in clinically normal dogs. Although ex vivo thrombin stimulation significantly increased P-selectin expression in platelets and platelet-leukocyte aggregates from basal levels, ULDAsp did not significantly affect thrombin-stimulated platelet P-selectin expression. Similarly, humans receiving aspirin doses ≥1 mg/kg also have not had significant change in P-selectin expression.

Measuring thrombin-induced P-selectin expression and platelet-leukocyte aggregate formation would likely not yield useful information in evaluating aspirin-treated dogs with IMHA. In contrast, ULDAsp treatment induced alterations in platelet aggregation variables as measured by whole blood impedance aggregometry in most dogs in our study. Similarly, impaired platelet aggregation response in the absence of flow cytometric changes has been reported for people treated with aspirin.

We pursued investigation of canine platelet P-selectin expression to evaluate its potential as an indicator of therapeutic response to ULDAsp because
an earlier study implicated P-selectin as a marker of platelet hyperactivity in dogs with IMHA. That work suggested that platelets in many of these patients have high basal P-selectin expression and have exaggerated P-selectin expression after in vitro phorbol myristate acetate stimulation; platelet aggregation responses were not investigated. Although the whole blood cytometric assay used in the study reported here is potentially applicable to measure basal platelet P-selectin expression in dogs with IMHA, the protocol has not been evaluated with phorbol myristate acetate as an agonist to test stimulated platelet response. We chose thrombin, rather than phorbol myristate acetate, reasoning that thrombin was a more physiologic agonist in stimulating platelet P-selectin expression and platelet-leukocyte aggregates, compared with phorbol myristate acetate. Phorbol myristate acetate mediates platelet aggregation through the protein kinase C pathway; invokes unique platelet cytoskeletal changes, and strongly augments granulocyte platelet aggregation. It would require additional studies to determine whether ULDAsp modulates phorbol myristate acetate-induced α-granule protein release.

Platelets adhere to leukocytes through binding of platelet P-selectin to a P-selectin glycoprotein ligand. This interaction enhances platelet aggregation by increasing platelet secretion of thromboxane and neutrophil production of platelet activating factor. In fact, formation of platelet-leukocyte aggregates is a marker of platelet activation and represents an important mechanism whereby leukocytes contribute to thrombus and platelet-leukocyte formation. Binding of leukocytes with platelets invokes expression of proinflammatory cytokines, oxidative burst, and surface expression of receptors enhancing inflammatory responses and cellular platelet aggregation.

Indeed, interaction of platelets and leukocytes is involved in diverse disease processes (eg, glomerulonephritis, vasculitis, and hepatitis), likely including IMHA in dogs. In humans, high-dose aspirin (15 to 60 mg/kg) has shown a dose-dependent inhibition of selective major platelet receptors and has been proposed to suppress platelet-leukocyte aggregate formation. Lower aspirin doses (1 to 7 mg/kg, PO, q 24 h) failed to alter platelet-leukocyte aggregate formation in humans. Short-term treatment of clinically normal dogs with ULDAsp did not attenuate formation of platelet-leukocyte aggregates. If platelet-leukocyte interactions are central to thromboembolic events in dogs with IMHA, a higher dose of aspirin, clopidogrel, or aspirin-clopidogrel combination might be appropriate. It remains unknown whether ULDAsp may have a more notable effect in dogs with systemic inflammation where enhanced molecular signaling and amplified receptor expression exist. Because platelet-leukocyte interactions are recognized components of inflammation and tissue repair, careful assessment of the effect of aspirin in clinically ill dogs will be necessary to assess its therapeutic role.

Because an ideal prophylactic antithrombotic agent for dogs with IMHA has yet to be identified, we previously investigated a treatment protocol combining glucocorticoids, azathioprine, and ULDAsp that seemed to improve survival. Although we did not precipitate adverse effects with ULDAsp, caution is warranted when treating dogs with systemic illnesses with aspirin. Depending on dose, aspirin can substantially influence multiple organs by inhibiting cyclooxygenase-dependent systems. Because aspirin imposes a dose-dependent inhibition on prostacyclin activity in various cells, careful dose titration is necessary to preserve beneficial vasodilatory and thromboprotective effects of prostacyclin. Aspirin at higher doses than used in the study reported here warrants concern in dogs because of potential detrimental enteric, renal, and hepatic effects. Our experience to date suggests ULDAsp minimizes iatrogenic complications while sustaining a modulatory influence on platelet aggregation in many dogs. On the basis of the findings of our present study and our clinical investigation in dogs with IMHA, we proffer that ULDAsp may reduce risk of thromboembolic complications without the untoward systemic effects associated with higher-dose aspirin administration. Aspirin is an important antithrombotic agent in thromboembolic disorders associated with heightened platelet reactivity because a single dose can impair platelet aggregation for up to 1 week, the approximate life span of a platelet. Even very low-dose aspirin, such as used in the study reported here, can alter platelet function. We tested platelet aggregation responses 24 hours after 2 days of ULDAsp administration but did not investigate the longevity of aspirin effects on platelet activity. It is important to consider that the antithrombotic influence of aspirin may be modulated by an accelerated rate of platelet turnover, as encountered in some dogs with IMHA. Thus, our findings should not be extrapolated to intermittent (eg, every other day administration) dosing with ULDAsp in dogs with IMHA. Prior studies in dogs have characterized this species as aspirin-resistant because approximately 70% of dogs have platelets unresponsive to an in vitro thromboxane agonist. Those findings are disparate with the results of this study, where we found that treatment with ULDAsp had an effect on platelet function in most dogs.

In our hospital, thrombi in dogs with IMHA have been confirmed at necropsy in a variety of vascular beds, including both the venous (pulmonary, splenic, portal, and limb) and arterial (pulmonary, aortic, iliac bifurcation, and limb) circulations. Although these thrombi seemingly may emanate from vessels with either low or high shear, thromboses of pulmonary and splenic vasculature are most commonly reported for dogs with IMHA. Identification of thrombi in those organs, however, may reflect the notable clinical signs associated with pulmonary thrombi or the ease of detection of splenic thrombi with ultrasonographic imaging. Segregation of involved vasculature on the basis of whether it maintains low or high shear is further complicated by the fact that vascular beds may be transformed by disease processes (eg, splenomegaly associated with marked extramedullary hematopoesis and vasocstriction reflecting systemic vasomotor responses). Additionally, the roles of systemic inflammation and vascular hypoxia on thromboembolism associated with IMHA in dogs remain to be determined. Thus, targeting prophylactic antithrombotic treatment on the basis of the anticipated shear conditions of affected vascular...
ture may not be straightforward. It is clear that many dogs with IMHA have abnormalities in their coagulative status, suggesting heightened coagulability and disseminated intravascular coagulation. The complexity of coagulative mechanisms and vascular shear–related effects responsible for pathological thrombi formation predicts that a single prophylactic treatment protocol will likely not protect all dogs with IMHA from thromboembolic complications.

Multiple mechanisms may contribute to the possible beneficial survival effect observed with ULDAsp combined with azathioprine and glucocorticoids in dogs with IMHA. Certainly, an antithrombotic influence seems plausible and is supported by our findings of attenuated platelet aggregation in most clinically normal dogs treated with ULDAsp. In addition to cyclooxygenase inhibition, the antithrombotic effects of aspirin in humans may represent interactive mechanisms such as modulation of cytokine (actions or signaling), effects on vascular endothelium or other blood cell components (including erythrocytes), or changes in activation signals. Our flow cytometry findings underscore the reality that physiologic responses may not correlate with receptor signals. On the basis of these results, flow cytometry interrogation of P-selectin expression would not be warranted for monitoring the influence of ULDAsp on platelet degranulation and activation in clinically normal dogs. Whether ULDAsp imparts anti-inflammatory effects and thereby reduces P-selectin expression in dogs with IMHA remains unknown. The results of whole blood impedance aggregometry for assessment of platelet aggregation in clinically normal dogs treated with ULDAsp indicate platelet inhibitory effects; however, additional studies are needed in dogs with IMHA (the clinical population of interest). If the benefits of ULDAsp result from a reduction in platelet-mediated hemostatic plug formation, a relationship between positive clinical outcome and in vitro platelet aggregation response should be detectable. Our study provides assay systems capable of detecting effects of ULDAsp treatment in dogs on platelet aggregation via whole blood impedance aggregometry, provided samples are collected and managed as described and analyzed within 8 hours.

References

21. Johnson GJ, Leis LA, King RA. Thromboxane responsiveness of...


35. Brooks MB, Randolfth JF; Warner K, et al. Evaluation of platelet function using novel tests to detect platelet procoagulant deficien-


46. Hwang DH. Species variation in platelet aggregation. In: Longe-


51. Rand ML, Perry DW, Packham MA, et al. Conditions influencing release of granule contents from human platelets in citrat-


55. Klinkhardt U, Harder S. Flow cytometric measurement of plate-

56. McKenzie ME, Malinin AI, Bell CR, et al. Aspirin inhibits sur-


60. Rand ML, Perry DW, Packham MA, et al. Conditions influencing release of granule contents from human platelets in citrat-

61. Santos MT, Valles J, Marcus AJ, et al. Enhancement of platelet reactivity and modulation of eicosanoid production by intact face glycoprotein IIb/IIIa, P-selectin, CD-63, CD-107a recep-

62. Hoit BD, Black WC, et al. Human platelet reaction to thrombin with aspirin or aspirin/dipyridamole combination in heartworm-negative, heartworm-infected, and embolized heart-

63. Lopez-Farre A, Caramelo C, Esteban A, et al. Effects of aspirin on platelet-leukocyte aggregates in whole blood by use of flow cyto-

64. Nieswandt B, Watson SP . Platelet-collagen interaction: is GPVI


67. Quinton TM, Dangelmaier C, Daniel JL, et al. Glycoprotein VI-


