
ORIGINAL ARTICLE

Platelet Size, Platelet Surface-Associated IgG, and Reticulated Platelets in Dogs with Immune-Mediated Thrombocytopenia

Melinda J. Wilkerson, DVM, MS, PhD; Wilma Shuman; Shannon Swist; Kenneth Harkin, DVM; James Meinkoth, DVM, PhD; A. Alan Kocan, PhD

Abstract: Immune-mediated thrombocytopenia (IMT) is a disorder in which bound IgG on the surface of platelets results in platelet removal and alterations in mean platelet volume. Using flow cytometry, alterations in platelet size, platelet surface-associated IgG (PSAIgG), and numbers of reticulated platelets were determined in 13 dogs with primary IMT and 4 dogs with secondary IMT induced by experimental infection with *Babesia gibsoni*. Effects of sample age on platelet parameters also were determined, using samples from 20 dogs with normal platelet counts analyzed within 4 hours and after 24, 48, and 72 hours of storage in EDTA. No significant changes in platelet count, platelet size, or reticulated platelet percentage were observed in samples assayed within 4 and 24 hours of blood collection; whereas PSAIgG values increased 3 to 7 fold in samples stored for 24-72 hours. Using reference values for freshly collected or 24-hour-old samples, 10 of 13 (77%) dogs with primary IMT and all *B gibsoni*-infected dogs had increased PSAIgG levels. In 12 (75%) of the 16 dogs with thrombocytopenia the percentage of reticulated platelets was increased; however, absolute numbers of reticulated platelets were within reference values. Moreover, PSAIgG level and the percentage of reticulated platelets were not always increased concurrently in dogs with primary and secondary IMT. Platelet microparticles were detected in all *B gibsoni*-infected dogs, 8 of 13 (62%) dogs with primary IMT, and transiently in a dog that responded to immunosuppressive treatment. The results of this study indicate that sample age and time of sampling during disease affect interpretation of platelet parameters in dogs with IMT. (*Vet Clin Pathol.* 2001;30:141-149) ©2001 American Society for Veterinary Clinical Pathology

Key Words: Platelet microparticles, platelet surface-associated antibody, reticulated platelets, thrombocytopenia

Canine thrombocytopenia frequently is immune-mediated, and occurs as a consequence of the removal of antibody-coated platelets by macrophages in the spleen and other tissues.¹ Immune-mediated thrombocytopenia (IMT) can be primary (autoimmune or idiopathic) or secondary due to infection, drugs, or neoplasia.² In primary IMT, platelet surface-associated antibody is often directed against platelet glycoproteins such as the fibrinogen receptor antigen gpIIb/IIIa.³ In secondary IMT, platelet destruction results from immune complexes bound to platelet Fc receptors, foreign antigens non-specifically adsorbed onto the platelet surface, or new antigens that are generated during the course of an infectious or neoplastic disease.² The principal antibody class associated with IMT is IgG.² Typically, dogs with IMT have normal to increased numbers of megakary-

ocytes. Decreased megakaryocytes in IMT may indicate that antibody bound to megakaryocytes is contributing to ineffective thrombopoiesis.⁴

Flow cytometric methods that evaluate a variety of platelet parameters in thrombocytopenic dogs have been established.⁵⁻⁷ The direct platelet-bound IgG assay is more sensitive than the serum platelet-bindable IgG assay because it detects antibodies on the platelet surface.⁸ The RNA-binding fluorescent dye thiazole orange can be used to identify newly synthesized (reticulated) platelets. This assay is rapid and less invasive than obtaining bone marrow samples and is reported to be useful in evaluating thrombopoiesis in dogs, horses, and human beings.^{7,9-11} Platelet microparticles have been detected by flow cytometry in human cases of idiopathic and secondary IMT.^{12,13}

Departments of Diagnostic Medicine and Pathobiology (Wilkerson, Shuman, Swist) and Clinical Sciences (Harkin), College of Veterinary Medicine, Kansas State University, Manhattan, Kans; and the Department of Pathobiology, College of Veterinary Medicine, Oklahoma State University, Stillwater, Okla (Meinkoth, Kocan). Corresponding author: Melinda J Wilkerson, DVM, PhD, Department of Diagnostic Medicine and Pathobiology, Kansas State University, 1800 Denison Ave, Manhattan, KS 66506 (e-mail: wilkersn@vet.ksu.edu).

We are unaware of studies that concurrently assess platelet size, platelet surface-associated antibody, and numbers of reticulated platelets in dogs with thrombocytopenia. The purpose of this study was to quantitate and compare these platelet parameters in dogs with primary and secondary IMT and in healthy dogs at various times after sample collection and at sequential intervals following the development of thrombocytopenia.

Materials and Methods

Dogs and blood samples

Whole blood samples were collected in EDTA Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ, USA) from 17 dogs with thrombocytopenia (platelet count of $<200,000/\mu\text{L}$) and 20 clinically healthy dogs (platelet counts of $>200,000/\mu\text{L}$). Of the thrombocytopenic dogs, 13 had primary IMT and 4 had secondary IMT. Criteria for primary IMT included the absence of an underlying etiology or concurrent disease, negative antibody titers to tick-borne diseases (*Erhlichia canis*, *Rickettsia rickettsii*, and *Borrelia burgdorferi*), and an increased platelet count in response to corticosteroid therapy. Three of the 13 dogs with primary IMT had systemic lupus erythematosus based on a positive anti-nuclear antibody titer and supportive clinical signs. Two of the 13 dogs had concurrent immune-mediated hemolytic anemia based on a positive Coombs' test and spherocytosis. Of the 13 dogs with primary IMT, 7 were admitted to the Veterinary Teaching Hospital at Kansas State University, where samples were analyzed within 4 hours of blood collection. One dog had a second blood sample taken after 1 month of treatment with prednisone (2mg/kg PO q12h). Whole blood samples from 6 of the dogs with primary IMT were submitted by outside veterinarians. These samples were shipped on ice and assayed within 24 hours of collection.

The 4 dogs with secondary IMT had been experimentally infected with *B gibsoni*.¹⁴ The dogs were mixed-breed 6-month-old littermates acquired and housed by the Laboratory Animal Resources unit at Oklahoma State University in accordance with National Institutes of Health guidelines for the care and use of laboratory animals. All experimental procedures were approved by the Institutional Animal Care and Use Committee, Oklahoma State University. Blood samples were obtained on day 0, and days 7, 15, 21, 28, and 42 postinfection. Experimentally infected dogs included a chronic carrier of *B gibsoni* (infected 60 days prior to this study), a clinically normal dog, a normal splenectomized dog, and a dog that had been inoculated 3 months prior to the current study but did not develop detectable parasitemia. Samples from a noninfected age- and breed-matched

dog also were collected.

The 20 clinically healthy dogs had been admitted to the Veterinary Teaching Hospital for routine vaccinations and elective surgeries. Criteria for selection were normal physical examination findings and CBC results. Samples were analyzed within 4 hours after arrival at the Clinical Immunology Laboratory at Kansas State University and after 24, 48, and 72 hours of storage at 4°C.

Platelet preparation

Platelet numbers were determined on whole blood samples using an automated hematology analyzer. A Cell-Dyn analyzer (Abbott Laboratories, Abbott Park, Ill, USA) was used at Oklahoma State University to determine platelet counts in dogs infected with *B gibsoni*. A Coulter S-IV (Hialeah, Fla, USA) was used at Kansas State University for platelet counts on all other samples. Manual platelet counts were performed with a Neubauer hemocytometer on all dogs with platelet counts of $<100,000/\mu\text{L}$. Platelet-rich plasma was prepared from blood samples as previously described.⁶ Plasma proteins were removed by 3 washes in Tyrode's buffer (0.14 M NaCl, 0.003 M KCl, 0.005 M NaH_2PO_4 , 0.001 M NaHCO_3 , 0.006 M Na_2EDTA , pH 7.1; Sigma Chemical Co, St. Louis, Mo, USA). Platelets were resuspended to a concentration of approximately 10^6 cells/mL in Tyrode's buffer.

Flow cytometric analysis

Platelet suspensions were analyzed using a FACScan (Becton Dickinson, San Jose, Calif, USA) interfaced with a 7600 Power Macintosh computer and Cell Quest software (Becton Dickinson). To determine the normal size distribution of platelets and to exclude RBCs, WBCs, and debris, a live gate (region 1) was placed on unstained platelets in a scatter plot using side and forward light scatter (SSC and FSC, respectively) properties.

Platelet size determination

Size ranges for platelets based on SSC and FSC were determined previously using latex beads ranging in size from 0.1 to 9.0 μm in diameter (Polybead polystyrene, Polysciences, Warrington, Penn, USA).¹⁵ In normal dogs, $>95\%$ of platelets were detected between 40 and 800 log channels on FSC, a subset of region 1 (region 2) corresponding to a size range of 1-3 μm in diameter. Large platelets fell in the high end of region 2 (800-1000 FSC log channels, 3-5 μm diameter). Using these electronic settings, RBCs could be detected in region 3 (6-10 μm , >1000 FSC log channels) if there was RBC contamination of the platelet-rich plasma. Platelet microparticles

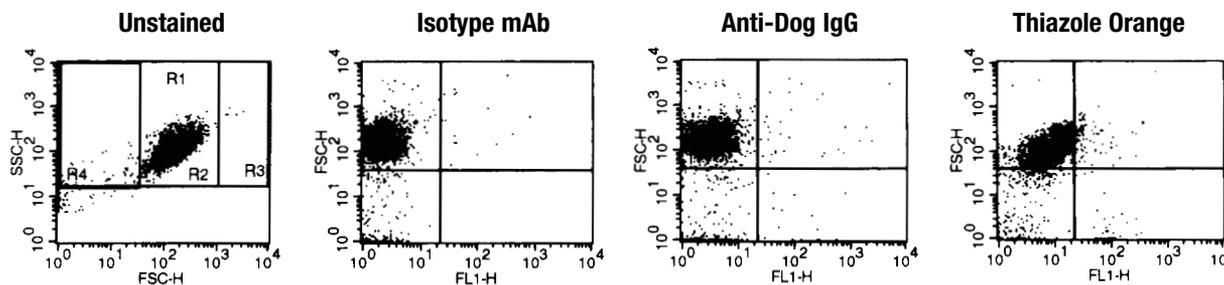


Figure 1. Bivariate dot plots of normal dog platelets separated from fresh EDTA blood samples. The left plot shows the size distribution of unstained platelets based on side (SSC-H, complexity) and forward (FSC-H, size) light scatter properties. Region 1 (R1) represents the live gate that defines platelets and incorporates regions 2, 3, and 4 (R2, R3, R4). Platelets of normal size (1-3 μm) and large size (3-5 μm) fall in R2. Region 3 contains RBCs (6-10 μm), and R4 contains platelet microparticles (<1 μm). The isotype mAb plot shows platelets probed with FITC-conjugated mouse IgG (isotype control). The anti-dog IgG plot shows platelets labeled with FITC-conjugated mouse anti-dog IgG. The far right plot indicates platelets stained with thiazole orange (reticulated platelets). PSAIgG- and thiazole orange-positive cells are shifted on the FL1-H axis into the right upper and lower quadrants.

(<1 μm diameter) were located in region 4 and expressed as a percentage of total platelets. For each 100 μL of platelet suspension, data were collected on 10,000 cells in the platelet gate (region 1). To determine whether microparticles had CD61, a platelet surface molecule, anti-CD61 antibody (0.62 $\mu\text{g}/\text{mL}$, RUU-PL7F12, Becton Dickinson) was used to stain normal samples.

Platelet surface-associated IgG (PSAIgG)

Fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody (mAb) to canine IgG (12.5 $\mu\text{g}/\text{mL}$, mouse anti-dog IgG, Sigma) was incubated with 100 μL of platelet suspension for 30 minutes. Background fluorescence was detected using platelets similarly incubated with an isotype control antibody (FITC-conjugated isotype-specific mouse IgG, Sigma). Platelet surface-associated IgG (PSAIgG) values were corrected by subtracting background fluorescence. The percentage of platelets in thrombocytopenic samples that were coated with IgG was defined by the total cells in region 1 that shifted into fluorescence (FL1)-positive quadrants. The vertical axis of the quadrant for thrombocytopenic samples was established on the FL1 axis \pm 2SD from the mean PSAIgG value for the 20 normal samples. The established quadrant setting was used as a template for all patient samples. Intra-assay precision of the PSAIgG test was determined by performing 5 sequential flow cytometric analyses on 8 normal dog samples.

Reticulated platelets

Platelet suspensions (100 μL) were mixed with 0.5 mL of thiazole orange (Becton Dickinson), incubated in the dark at 20°C for 30 minutes, and analyzed using the

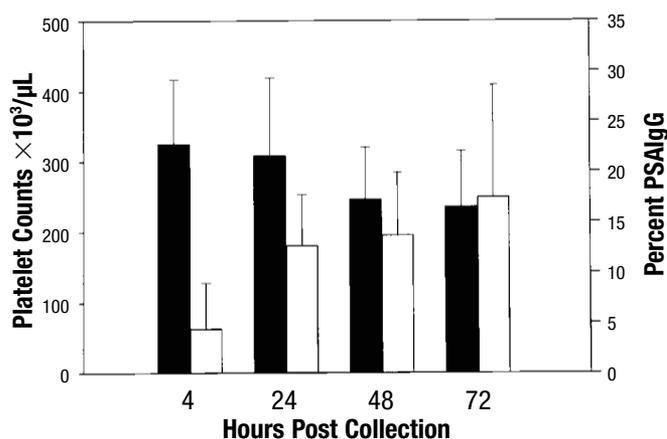


Figure 2. Effect of time and storage on platelet counts (solid bars) and percentage of platelets coated with platelet surface-associated IgG (PSAIgG) (open bars) in EDTA whole blood samples from 20 normal dogs. Platelets were isolated within 4 hours of collection and after 24, 48, and 72 hours of storage at 4°C. Bars are mean \pm SD.

argon laser of the FACScan flow cytometer. Platelets labeled with thiazole orange were identified by a shift in FL1 fluorescence. Background fluorescence was determined using unstained platelets without thiazole orange. The vertical axis of the quadrant for thrombocytopenic samples was established on the FL1 axis \pm 2 SD from the mean reticulated platelet value for 20 normal samples. This quadrant setting served as a template for all patient samples. The absolute number of reticulated platelets was determined by multiplying the percentage of reticulated platelets by the total number of platelets in whole blood. Intra-assay precision of the reticulated platelet test was determined by performing 5 sequential flow cytometric analyses on 8 normal dog samples.

Platelet Alterations in Dogs with Immune-Mediated Thrombocytopenia

Table 1. Results of platelet analyses on blood samples from thrombocytopenic dogs analyzed within 4 hours of collection.

Dog No.	Breed	Age (yrs)	Sex*	Platelet Count (cells/ μ L)	PSAIgG [†] (%)	Reticulated Platelets		Microparticles (%)	Diagnosis [‡]
						%	cells/ μ L		
1	Rottweiler	10	FS	2000	15	10	200	76	Primary IMT, IMHA
2	Irish Setter	4	MN	14,000	15	19	2660	16	Primary IMT
3	Welsh Corgi	8	FS	28,000	20	72	20,160	28	Primary IMT, IMHA
4	Standard Poodle	7	FS	121,000	81	6	7260	0	Primary IMT
5	Doberman Pinscher	7	FS	1000	5	10	100	81	Primary IMT
6	Shetland Sheepdog	8	FS	4000	7	50	2000	18	Primary IMT
7	Shetland Sheepdog	12	MN	87,000	2	31	26,970	27	Primary IMT, SLE
Reference values [§]				>200,000	<14	<7	<14,000	<15	

*FS indicates spayed female; MN, neutered male.

[†]PSAIgG indicates the percentage of platelets coated with platelet surface-associated IgG.

[‡]IMT indicates immune-mediated thrombocytopenia; IMHA, immune-mediated hemolytic anemia; SLE, systemic lupus erythematosus.

[§]Calculated as mean \pm 2SD using values from 20 healthy dogs.

Statistical analysis

Variance in the results of the PSAIgG assay over time (4, 24, 48, and 72 hours after blood collection from normal dogs) was compared using Bonferroni's 95% simultaneous confidence interval. Differences over time in platelet counts and in the percentage of platelets with PSAIgG were compared using the random slopes model (SAS version 8.1, SAS Institute, Research Triangle Park, NC, USA). This model used linear regression to determine the slope of PSAIgG percentage and platelet count over time for each sample, and a 1-tailed *t*-test to compare the mean slopes. The null hypothesis was that PSAIgG values and platelet counts do not change over time, resulting in mean slopes of 0. This hypothesis was rejected if the mean slope of the platelet counts was significantly <0 or the mean slope of the PSAIgG values was significantly >0 ($P < .05$).

Results

Normal test parameters

Flow cytometric criteria were established using samples from normal dogs (Figure 1). Platelet microparticles were located in region 4 of the unstained platelet scatter plot. Platelets that reacted with FITC-conjugated antibody (isotype control and mAb to dog IgG) or thiazole orange resulted in a shift to the right on the FL1 axis.

Effect of storage on normal platelet values

The percentage of platelet microparticles was low in

normal samples assayed within 4 hours (mean \pm SD, 6.0% \pm 4.0%) and 24 hours (9.6% \pm 3.9%) of blood collection. Less than 5% of platelet microparticles in samples from normal dogs reacted with anti-CD61 antibody (data not shown). There was variable CD61 staining of normal platelets among individual dogs and between 4-hour (71.2% \pm 13.7%) and 24-hour (53.8% \pm 29.8%) samples. Based on these and previous results in which decreased CD61 was detected on stored or activated platelets independent of platelet microparticle formation,¹⁵ CD61 antibody was not used to define the platelet gate.

Platelet counts in samples from normal dogs were stable for up to 24 hours after collection (Figure 2); thereafter, the counts declined (slope = -5.468, $P < .001$). The decrease in platelet count in many 48- and 72-hour-old samples was attributed to platelet clumping, as determined by evaluation of blood smears. The percentage of platelets with surface IgG increased 3- to 7-fold in samples stored for 24 (12.6% \pm 5.0%), 48 (13.6% \pm 6.3%), and 72 hours (17.4% \pm 11.2%) compared with samples analyzed within 4 hours of collection (4.4% \pm 4.6%) (Figure 2). The increase in PSAIgG values in samples over time was significant (slope = 4.549, $P < .001$). The variance in PSAIgG values for 4-hour samples was significantly less than that of stored samples ($P < .01$). Reference values for the percentage of platelets with PSAIgG were defined as $<14\%$ and $<23\%$ for samples processed within 4 and 24 hours of blood collection, respectively, based on the mean \pm 2SD of results.

The percentage of reticulated platelets in normal dogs did not differ between 4-hour (2.7% \pm 2.0%) and 24-hour (2.6% \pm 1.5%) samples. The normal cutoff value was designated as $<7\%$ based on the mean \pm 2SD of results

Table 2. Results of platelet analyses on blood samples from thrombocytopenic dogs analyzed within 24 hours of blood collection.

Dog No.	Breed	Age (yrs)	Sex*	Platelet Count (cells/ μ L)	PSAIgG [†] (%)	Reticulated Platelets		Microparticles (%)	Diagnosis [‡]
						%	cells/ μ L		
8	English Cocker	11	FS	1000	46	8.5	9	94	Primary IMT
9	English Setter	4	FS	5000	32	6	300	13	Primary IMT
10	Schnauzer	6	FS	14,000	31	Not done	Not done	3	Primary IMT
11	Dachshund	8	FS	29,000	48	12	3480	12	Primary IMT, SLE
12	Labrador Retriever	11	M	69,400	32	28	19,432	18	Primary IMT
13	Golden Retriever	1	FS	189,000	34	25	47,250	14	Primary IMT, SLE
Mixed-breed [§]									
14	Splenectomized	.5	F	15,000	35	3	450	35	Secondary IMT
15	Infected	.5	F	25,000	28	5	5000	35	Secondary IMT
16	Reinfected	.5	F	35,500	39	20	7100	36	Secondary IMT
17	Chronic infection	.5	F	45,000	36	10	4500	74	Secondary IMT
Reference values [¶]				>200,000	<23	<7	<14,000	<18	

*FS indicates spayed female.

[†]PSAIgG indicates the percentage of platelets with platelet surface-associated IgG.

[‡]IMT indicates immune-mediated thrombocytopenia; SLE, systemic lupus erythematosus.

[§]Mixed-breed dogs experimentally infected with *Babesia gibsoni*. Values for platelet count, PSAIgG, and microparticles are the maximum values reached during infection. Values for reticulated platelets are those obtained on the day of peak PSAIgG levels.

[¶]Calculated as mean \pm 2SD using values from 20 healthy dogs.

for the 4-hour samples. Mean intra-assay coefficients of variation for PSAIgG and reticulated platelet values were 7% and 5%, respectively.

Platelet alterations in thrombocytopenic dogs

Samples from thrombocytopenic dogs analyzed immediately after collection had platelet counts ranging from 1000 to 121,000/ μ L (Table 1). Using 4-hour reference values for the PSAIgG assay, 4 of 7 thrombocytopenic dogs had an increased percentage of platelets with PSAIgG. All dogs with platelet counts of <100,000/ μ L had increased percentages of reticulated platelets and platelet microparticles. Although the percentage of reticulated platelets ranged from 6% to 72%, 5 of 7 dogs with primary IMT had absolute numbers of reticulated platelets within reference values. The dog (dog 4) with the highest platelet count and highest percentage of platelets with PSAIgG had no detectable platelet microparticles. Two dogs (dogs 1 and 5) with primary IMT were profoundly thrombocytopenic (platelet counts of 2000/ μ L and 1000/ μ L) and had the highest percentage of platelet microparticles.

All samples from dogs with primary IMT that were analyzed within 24 hours of collection had an increased percentage of platelets with PSAIgG (range, 31%-48%), regardless of wide variability in platelet count. The per-

centage of reticulated platelets also was increased in 4 of the dogs (Table 2). Two dogs had increased absolute numbers of reticulated platelets; the dog with the highest reticulated platelet count (dog 13) also had the highest platelet count (189,000/ μ L). The percentage of platelet microparticles in dogs with primary IMT (samples analyzed within 24 hours) was low (<20%) except for dog 8, which had 94% microparticles and 1000 platelets/ μ L.

Using bivariate dot plots, platelet assay results for dog 8 were compared in pre- and post-treatment samples (Figure 3). Dog 8 had profound thrombocytopenia with a predominance of platelet microparticles that were negative for CD61 and positive for PSAIgG (Figure 3A). In a second sample obtained after 1 month of prednisone treatment, the platelet count rose to 718,000/ μ L, platelets returned to normal size, and PSAIgG levels decreased to within reference values (Figure 3B). In contrast to the first sample, the majority of platelets in the second sample reacted with CD61 antibody. Although the mild increase in percentage of reticulated platelets in this dog did not reflect the thrombocytosis, the absolute reticulated platelet count (93,340/ μ L) was markedly increased over the reference value (<20,000/ μ L) after 1 month of treatment.

In samples from dogs with secondary IMT due to *B. gibsoni* infection, maximum PSAIgG values were ob-

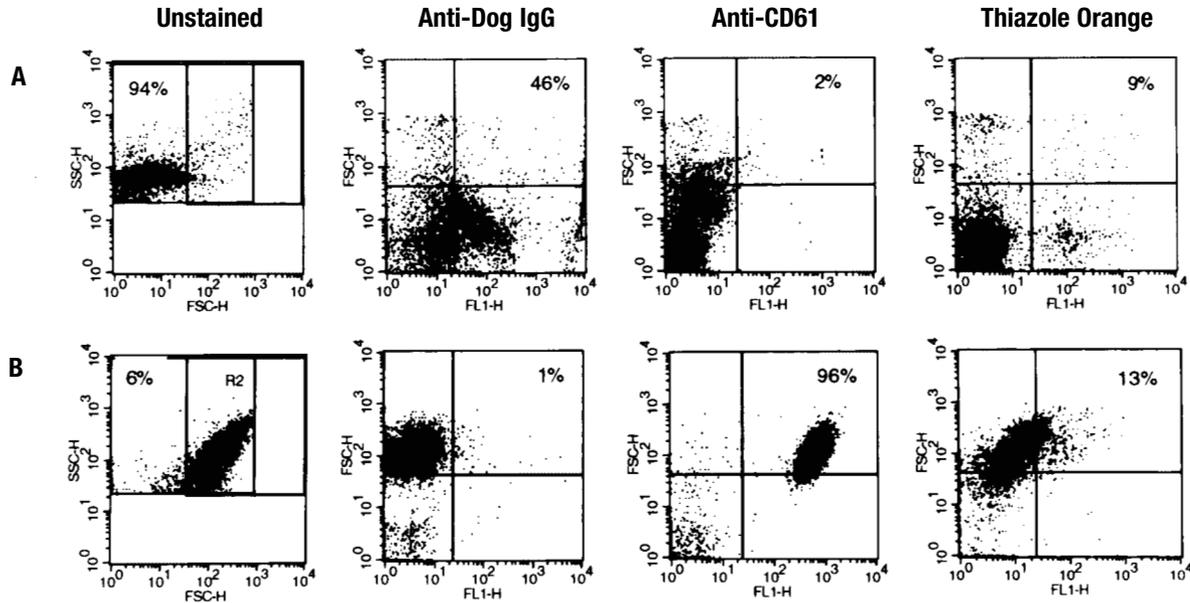


Figure 3. Bivariate dot plot analysis of platelets from dog 8, with primary immune-mediated thrombocytopenia prior to treatment (A) and after 1 month of treatment with prednisone (B). The left plots show the size of unstained platelets. Anti-dog IgG plots show the percentage of platelets labeled with IgG. Anti-CD61 plots show the percentage of platelets labeled with antibody to CD61. The far right plots show the percentage of thiazole orange-labeled platelets (reticulated platelets). From left to right, the numbers in each plot indicate the percentage of platelet microparticles, and the percentage of cells positive for PSAIgG, anti-CD61 mAb, and thiazole orange, respectively.

tained on day 15 (dogs 14 and 15, acute infection), day 21 (dog 16, reinfection), and day 0 (dog 17, chronic infection) (Table 2). Although 2 dogs had a normal percentage of reticulated platelets at the time of maximal PSAIgG levels, samples from subsequent days had an increased percentage of reticulated platelets (Figure 4). The percentage of platelet microparticles was 35%-36% at the time of maximal PSAIgG levels in all dogs except dog 17, which had a marked increase in platelet microparticles (74%).

Sequential platelet assays were done at defined intervals postinfection on dogs with secondary IMT (Figures 4 and 5). On day 0, the chronically infected dog had 45,000 platelets/ μ L and the reinfected dog, which had cyclic bouts of thrombocytopenia, had 27,000 platelets/ μ L. The percentage of platelet microparticles on day 0 was highest in the chronically infected dog (74%) and the reinfected dog (64%), and lowest in acutely infected dogs. Day 0 platelet counts for both the normal dog and the splenectomized acutely infected dog were near or above the normal cutoff of 200,000 platelets/ μ L. On day 0, the chronically infected dog had the highest PSAIgG level (36%), whereas all other infected dogs had normal PSAIgG levels. In the 2 acutely infected dogs, platelet numbers dropped to <50,000/ μ L by day 7 postinfection, and PSAIgG levels peaked at 28% and 35% by 15 days postinfection. In the reinfected dog, PSAIgG levels peaked at 39% by 21 days postinfection. PSAIgG levels

in all infected dogs decreased steadily after reaching peak values, and fell to within normal limits by either 28 or 42 days postinfection.

In contrast, reticulated platelets in all infected dogs steadily increased following peak PSAIgG values, in conjunction with rising platelet counts (Figure 4). The splenectomized dog had the highest reticulated platelet percentage, at day 28. However, the dog was euthanized on day 30 because of severe anemia (HCT=8%). Although the percentage of reticulated platelets in the uninfected control dog fluctuated, values remained <15%. The percentage of platelet microparticles in the chronically infected dog decreased during the observation period. In the reinfected dog, the percentage of platelet microparticles decreased to 17% followed by a gradual increase to 45% by the end of the study (Figure 5). In the splenectomized dog, platelet microparticle percentage increased to 37% by day 7 postinfection, followed by a gradual decline to 10% by day 30 postinfection. The other acutely infected dog had an increased percentage of microparticles at day 7 (36%) that persisted to the end of the study (42%). An uninfected control dog maintained normal platelet counts and PSAIgG and microparticle values throughout the study.

Discussion

Sample storage for \geq 24 hours affected PSAIgG values in

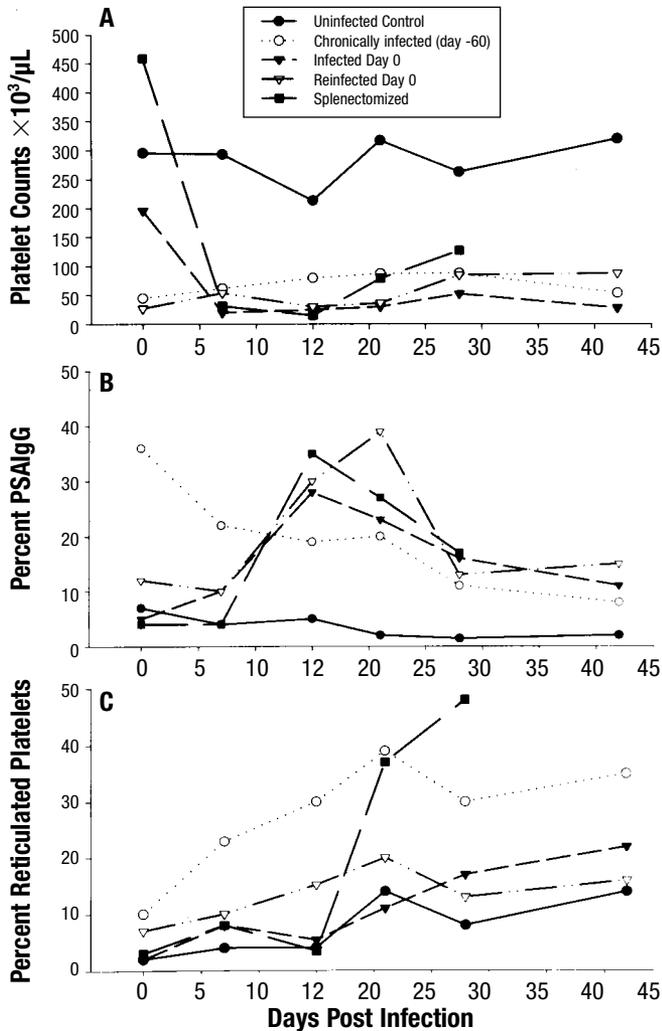


Figure 4. Platelet counts (A), percentage of platelets with PSAIgG (B), and percentage of reticulated platelets (C) in 4 dogs infected with *B gibsoni* and 1 uninfected control dog. Reference values are >200,000 platelets/μL, <23% platelets with PSAIgG, and <7% reticulated platelets.

normal dog platelets, whereas reticulated platelet values did not differ between fresh and 24-hour-old samples. Ten of 13 dogs with primary IMT and all dogs with secondary IMT had increased PSAIgG values. PSAIgG level and reticulated platelet percentage were not always increased concurrently in dogs with primary and secondary IMT. The percentage of reticulated platelets was increased more frequently than were absolute numbers, unless platelet counts were $\geq 50,000/\mu\text{L}$. The percentage of platelet microparticles in dogs with IMT was variable, but was highest in dogs with profound thrombocytopenia ($< 5000/\mu\text{L}$).

In contrast to a previous report that suggested PSAIgG levels could be reliably determined in samples stored up to 72 hours after collection,⁶ the present results

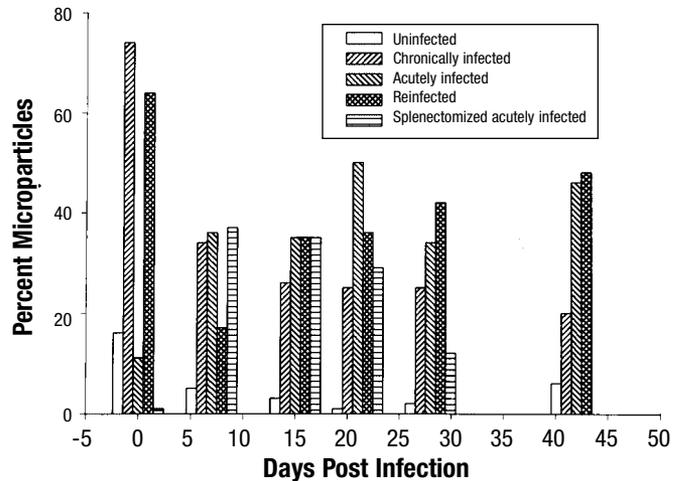


Figure 5. Percentage of platelet microparticles in *B gibsoni*-infected dogs during the course of infection.

showed increased PSAIgG values in stored blood samples compared with samples analyzed within 4 hours of collection. Therefore, we established separate reference values for the percentage of platelets with PSAIgG in freshly analyzed samples and in samples stored for 24 hours at 4°C. Samples analyzed within 4 hours after blood collection had the lowest background values for PSAIgG. Values obtained for 24-hour-old samples were considered valid only when interpreted in the context of 24-hour reference values. In a previous study using solid-phase ELISA, increased amounts of platelet-bound antibody also were detected in EDTA- and citrate-anticoagulated blood samples stored >24 hours.¹⁶

Although platelet-bound antibodies have been described in dogs with *B canis* infection,⁸ this study is the first to document PSAIgG levels in dogs with experimentally induced *B gibsoni* infection. In 3 infected dogs, PSAIgG levels increased transiently over a 2-week period during which platelet counts decreased. Similar findings were observed in dogs experimentally infected with *E canis* or *Rickettsia* (Rocky Mountain spotted fever), in which PSAIgG levels were elevated for a 20-day period that corresponded with the duration of thrombocytopenia.¹⁷

The significance of platelet microparticles in thrombocytopenic dogs is uncertain. Platelets from healthy dogs are typically 1-3 μm in diameter.¹⁵ In this study, the percentage of platelet microparticles tended to be higher in samples with low platelet counts. Platelet microparticles have been described in human beings with primary IMT and in patients undergoing long-term dialysis in which antibody complexes against heparin and platelet Fc receptors caused secondary IMT.^{12,13,18} As in the latter condition, canine platelet microparticles may form in response to the binding of either the Fab or Fc

portions of IgG to the platelet surface. IgG-induced platelet activation could explain why surface CD61 is lost from platelet microparticles, since distribution of CD61 can be altered on activated platelets.¹⁹ Platelet microparticles in dogs with profound thrombocytopenia could be important clinically because of the procoagulant properties of microparticles.^{20,21}

In previous studies, the reticulated platelet percentage was increased in dogs with naturally occurring IMT, but normal in dogs with carboplatin-induced suppression of thrombopoiesis.^{7,22} In the current study, we also detected an increased percentage of reticulated platelets in dogs with IMT; however, absolute numbers of reticulated platelets often were within reference values, principally because of the very low number of total platelets in many of the dogs.

Unlike the majority of dogs with primary IMT, dogs infected with *B gibsoni* did not have an increased percentage of reticulated platelets at the time of peak PSAIgG levels. Instead, reticulated platelet percentage increased approximately 1 week after peak PSAIgG levels. In chronically infected and splenectomized dogs, the reticulated platelet percentage peaked when platelet numbers exceeded 20,000/ μ L. Similar results were reported in human patients with IMT, in which normal or decreased numbers of reticulated platelets were seen when platelet counts were <20,000/ μ L.²³ In this study, we found increased absolute numbers of reticulated platelets in thrombocytopenic dogs with platelet counts of 50,000-100,000/ μ L. A possible explanation for this phenomenon is that reticulated platelets are destroyed as rapidly as are older platelets in IMT, particularly when platelet numbers drop to <20,000/ μ L.¹¹ In severely thrombocytopenic human patients, platelet life span is <3 days when the platelet count is 50,000/ μ L and 1 day when the count is <20,000/ μ L and PSAIgG concentration is high.^{24,25} This reduced life span of mature platelets approaches the time at which reticulated platelets would be indistinguishable from mature cells, since reticulated platelets lose RNA within 24 hours after entering the peripheral circulation.²⁶ Therefore, in cases of severe thrombocytopenia with high levels of PSAIgG, both mature and reticulated platelets may be equally susceptible to antibody-mediated removal.²⁶ Mouse models of IMT support this hypothesis. Absolute numbers of reticulated platelets in mice fell to low levels after injection of anti-platelet antiserum,²⁷ and numbers continued to be depressed as long as antibody-induced destruction of platelets continued. Only after bone marrow activity reached 3-5 times basal production and platelet numbers had risen significantly did reticulated platelet numbers increase.¹¹ Data from the *B gibsoni*-infected dogs indicate that reductions in PSAIgG levels contribute to increased platelet and reticulated platelet

numbers. Moreover, the rapid rise in total and reticulated platelet counts in the splenectomized infected dog shortly after PSAIgG values declined illustrates the important role of splenic macrophages in removing antibody-coated platelets from the peripheral circulation.

In this study, we used flow cytometry to measure and compare several platelet parameters in dogs with IMT. The results demonstrate that sample age and response to therapy are important factors to consider when interpreting platelet assay results. Tests that measure platelet microparticles, PSAIgG, and reticulated platelets are useful in the diagnosis and monitoring of IMT in dogs only if interpreted in the context of specific reference values established for freshly collected samples or samples analyzed within 24 hours of blood collection. Tests results are invalid if samples are older than 24 hours. \diamond

Acknowledgements

The authors thank Dr George Milliken and Oumar Sy for their advice regarding statistical methods and Derek Mosier for his critical review of the manuscript. This project was funded in part by a Companion Animal Grant from the College of Veterinary Medicine, Oklahoma State University.

References

1. Mackin A. Canine immune-mediated thrombocytopenia. *Compend Cont Educ Pract Vet.* 1995;17:353-362.
2. Lewis DC, Meyers KM. Canine idiopathic thrombocytopenic purpura. *J Vet Intern Med.* 1996;10:207-218.
3. Lewis DC, Meyers KM. Studies of platelet-bound and serum platelet-bindable immunoglobulins in dogs with idiopathic thrombocytopenic purpura. *Exp Hematol.* 1996;24:696-701.
4. Joshi BC, Jain NC. Experimental immunologic thrombocytopenia in dogs: a study of thrombocytopenia and megakaryocytopoiesis. *Res Vet Sci.* 1977;22:11-17.
5. Kristensen AT, Weiss DJ, Klausner JS, Laber J, Christie DJ. Comparison of microscopic and flow cytometric detection of platelet antibody in dogs suspected of having immune-mediated thrombocytopenia. *Am J Vet Res.* 1994;55:1111-1114.
6. Lewis DC, McVey DS, Shuman WS, Muller WB. Development and characterization of a flow cytometric assay for detection of platelet-bound immunoglobulin G in dogs. *Am J Vet Res.* 1995;56:1555-1558.
7. Weiss JD, Townsend E. Evaluation of reticulated platelets in dogs. *Comp Haematol Int.* 1998;8:166-170.
8. Lewis DC, Meyers KM, Callan B, Bucheler J, Giger U. Detection of platelet-bound and serum platelet-bindable antibodies for diagnosis of idiopathic thrombocytopenic purpura in dogs. *J Am Vet Med Assoc.* 1995;206:47-52.
9. Russell KE, Perkins PC, Grindem CB, Walker KM, Sellon DC. Flow cytometric method for detecting thiazole orange-positive (reticulated) platelets in thrombocytopenic horses. *Am J Vet Res.* 1997;58:1092-1096.

10. Kienast J, Schmitz G. Flow cytometric analysis of thiazole orange uptake by platelets: a diagnostic aid in the evaluation of thrombocytopenic disorders. *Blood*. 1990;75:116-121.
11. Ault KA. Flow cytometric measurement of platelet function and reticulated platelets. *Ann NY Acad Sci*. 1994;677:293-308.
12. Nomura S, Yanabu M, Fukuroi T, et al. Anti-phospholipid antibodies bind to platelet microparticles in idiopathic (autoimmune) thrombocytopenic purpura. *Ann Hematol*. 1992;65:46-49.
13. Lee DH, Warkentin TE, Denomme GA, Hayward CPM, Kelton JG. A diagnostic test for heparin-induced thrombocytopenia: detection of platelet microparticles using flow cytometry. *Br J Haematol*. 1996;95:724-731.
14. Kocan AA, Kjemtrup A, Meinkoth JW. A genotypically unique *Babesia gibsoni*-like parasite recovered from a dog in Oklahoma. *J Parasitol*. 2001;87:437-438.
15. Wilkerson MJ, Shuman W. Alterations in normal canine platelets during storage in EDTA anticoagulated blood. *Vet Clin Pathol*. 2001;30:107-113.
16. Lewis DC, Meyers KM. Effect of anticoagulant and blood storage time on platelet-bound antibody concentrations in clinically normal dogs. *Am J Vet Res*. 1994;55:602-605.
17. Grindem CB, Breitschwerdt EB, Perkins PC, et al. Platelet-associated immunoglobulin (antiplatelet antibody) in canine Rocky Mountain spotted fever and ehrlichiosis. *J Am Anim Hosp Assoc*. 1999;35:56-61.
18. Wenche JY, Horstman LL, Arce M, Ahn YS. Clinical significance of platelet microparticles in autoimmune thrombocytopenias. *J Lab Clin Med*. 1992;119:334-345.
19. Cramer EM, Savidge GF, Vainchenker W, et al. Alpha-granule pool of glycoprotein IIb-IIIa in normal and pathologic platelets and megakaryocytes. *Blood*. 1990;75:1220-1227.
20. Sims PJ, Faion EM, Wiedmer T, Shattil SJ. Complement proteins C5b-9 cause release of membrane vesicles from the platelet surface that are enriched in the membrane receptor for coagulation Va and express prothrombinase activity. *J Biol Chem*. 1988;263:18205-18212.
21. Warkentin TE, Hayward CPM, Boshkov KL, et al. Sera from patients with heparin-induced thrombocytopenia generate platelet-derived microparticles with procoagulant activity: an explanation for the thrombotic complications of heparin-induced thrombocytopenia. *Blood*. 1994;84:3691-3699.
22. DeHeer H, Grindem C, Russell K, Cullins I. Flow cytometric analysis of reticulated platelets in dogs. An investigation of platelet kinetics in thrombocytopenic disorders [abstract]. *Vet Pathol*. 1999;36:487.
23. Karpatkin S. Autoimmune thrombocytopenic purpura. *Blood*. 1980;56:329-343.
24. Ballem PJ, Segal GM, Stratton JR, et al. Mechanisms of thrombocytopenia in chronic autoimmune thrombocytopenic purpura: evidence of both impaired platelet production and increased platelet clearance. *J Clin Invest*. 1987;80:33-40.
25. Heyns A, Badenhorst PN, Lotter MG, et al. Platelet turnover and kinetics in immune mediated thrombocytopenic purpura: results with autologous ¹¹¹In-labeled platelets and homologous ⁵¹Cr-labeled platelets differ. *Blood*. 1986;67:86-92.
26. Dale GL, Riese P, Hynes LA, Burstein SA. Demonstration that thiazole-orange-positive platelets in the dog are less than 24 hours old. *Blood*. 1995;85:1822-1825.
27. Ault KA, Rinder HM, Mitchell J, et al. The significance of platelets with increased RNA content (reticulated platelets): a measure of the rate of thrombopoiesis. *Am J Clin Pathol*. 1992; 98:637-646.