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Canine Cyclic Hematopoiesis: Platelet Size and Thrombopoietin Level in Relation to Platelet Count[1] (39561)

T. P. McDONALD, ROSE CLIFT, AND J. B. JONES

University of Tennessee Memorial Research Center, Center for the Health Sciences, Knoxville, Tennessee 37920

Data from several laboratories have indicated that young platelets are larger and heavier than other platelets (1-7). Several workers (1-6) have shown that these platelets become smaller and lighter with age. However, in one study (7), it was claimed that large platelets produced following acute blood loss did not decrease in volume as they aged. These workers proposed that large platelets obtained from acutely bled dogs are "stress" platelets and may represent an aberrant population. Biochemical data support the finding of a decrease in platelet size with age (8). However, kinetic data neither absolutely prove that large-heavy platelets are the only young platelets nor rule out the possibility that large-heavy platelets might be "stress" platelets.

Cyclic hematopoiesis (CH) in dogs has been extensively studied in several laboratories (9-11). This syndrome in dogs serves as a model for studying cyclic thrombopoiesis disorders in humans (12, 13). Previously it has been established that in CH dogs cycling of platelets occurs at 11- to 14-day intervals and that platelet values cycle from near normal to above normal values (14). Since platelet counts cycle in these dogs and platelet size is thought to be related to the age of an individual platelet, it seemed possible that average platelet size might fluctuate with changing age composition of the platelet population as the proportion of young platelets changed.

Plasma levels of a thrombopoiesis-stimulating factor (TSF or thrombopoietin), a humoral agent that controls blood platelet production, were shown to fluctuate in a patient with cycling thrombopoiesis (13). This worker presented evidence in favor of the hypothesis that TSF deficiency may cause platelet counts to cycle in humans. In an attempt to clarify the causes of cycling platelet counts in CH dogs, TSF measurements were made on plasma fractions.

Materials and methods. Littermate CH dogs (Nos. 489, 490, 491) and a normal control littermate dog (No. 492) were used in the sizing studies. Throughout the study, less than 3 ml of blood were taken per day from these dogs (weighing about 6 kg at the beginning of the experiment). In another study, a CH dog (No. 347, weighing 10.5 kg) and a normal control dog (No. 415, weighing 10.9 kg) were used to determine the TSF levels associated with thrombopoiesis, and in these dogs 30 ml of blood were collected each day.

Platelet counts (determined by direct counting under phase-contrast microscopy) were made on blood taken from the jugular vein into vacutainers containing EDTA. For size measurements, platelets were taken from other samples of blood diluted into heparin. Approximately 0.5 ml of blood from each dog was transferred into a plastic tube (12 × 75 mm) containing 1 ml of a saline-heparin solution (100 units heparin), and the platelet-rich plasma (PRP) was separated by centrifugation at 450*g* for 4.5 min at 22°. The PRP was then removed and diluted with Isoton (Coulter) to a concentration of 12,000 to 18,000 platelets/100 μl for size analysis.

Average platelet sizes on a logarithmic scale were determined by use of an ElectroZone/Celloscope (Particle Data, Inc.) equipped with a 128-channel analysis accessory and direct readouts to an X-Y oscilloscope, and an X-Y plotter. The instrument settings were: log 10 (logarithmic span of about 10 doublings of particle volume or a 10:1 in diameter); current, $2^{1/2}$; and gain, 17. The multichannel analyzer was set for acquisition to a count of 4000 in

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the peak channel. Calibration was maintained at all times via frequent checking with particles of known size. Triplicate platelet samples were sized, size distributions were plotted by use of the X-Y plotter, and size (microns diameter) was calculated as previously described (15).

In the TSF study, CH dog No. 347 and a suitable control dog (No. 415) were bled from the jugular vein at daily intervals for 5 days. In all cases, a standard volume of 30 ml of blood was collected from each dog into a conical centrifuge tube containing 1 ml of citrate-phosphate-dextrose (CPD) solution (citric acid, 4.57 g; sodium citrate, 36.8 g; NaH_2PO_4, 3.1 g; dextrose, 35.66 g/liter of water). The plasma from each dog was then subjected to fractionation using DEAE-phosphate cellulose column chromatography (16). This technique has been shown to be useful for concentrating the TSF. Prior to chromatography, the plasma from each bleeding of the dogs was dialyzed for 18 hr against three changes of distilled water, adjusted to pH 5.5, and then added to a column containing DEAE-phosphate cellulose, pH 5.5. The TSF-rich fraction was eluted from the column with 1.0 *M* NaCl in 0.15 *M* Na_2HPO_4 buffer, pH 8.2 (Step I dog plasma TSF). After elution, the fraction was dialyzed against distilled water and lyophilized to dryness.

The lyophilized Step I dog plasma was resuspended into saline and assayed for TSF content by use of thrombocythemic mice (17). Five days before injection of the Step I dog plasma preparations (extracted from CH and normal dog plasmas), the assay mice were given a single ip injection of rabbit anti-mouse platelet serum (RAMPS). RAMPS was produced in rabbits as described previously (18). This antiserum produced a characteristic thrombocytopenia at 3 to 4 hr which was followed by rebound thrombocytosis. While thrombocythemic, mice were injected sc with 20 mg of protein of Step I dog plasma preparations, two times on Day 5 after the RAMPS injection, and two times again on Day 6. Thirty microCuries of Na_2 $^{35}SO_4$ in 0.5 ml of saline were injected iv on Day 7 and the percentage of ^{35}S-incorporation of injected dose into circulating platelets was measured 24 hr later (Day 8) in blood samples obtained by cardiac puncture.

The platelet ^{35}S incorporation data and platelet size measurements were subjected to Student's *t* test.

Results. Figure 1 shows the results of de-

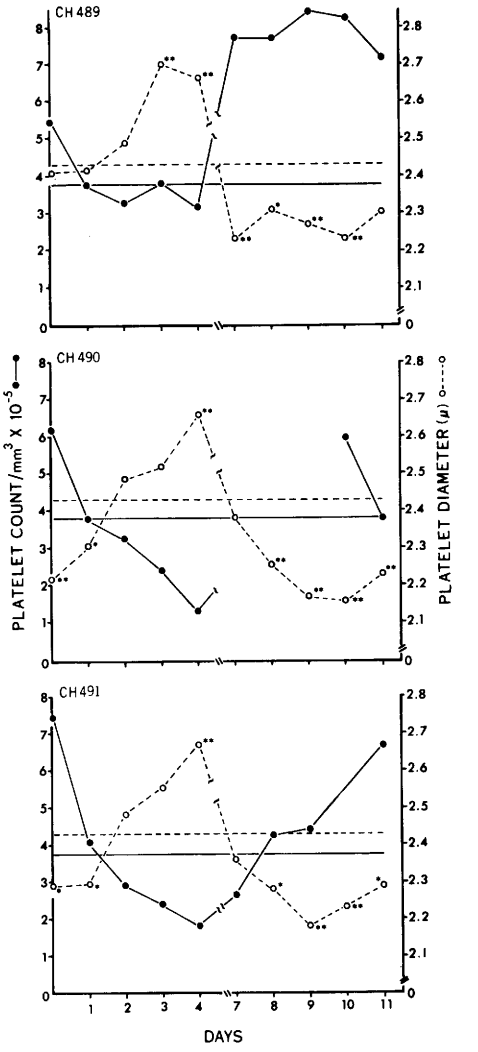

FIG. 1. Platelet counts and platelet sizes of four littermate dogs measured during a 2-week period: CH dogs, No. 489, No. 490, and No. 491, and a normal dog, No. 492. Horizontal dashed lines represent average platelet size and the solid lines show the average platelet count for the normal dog. Platelet sizes for the normal dog varied from 2.34- to 2.49-μm diameter; platelet counts were from 3.12 to 4.0 $\times$ 10^5/mm^3 for the 2-week period. Platelet sizes were significantly different from normal control: $^*P < 0.005$; $^{**}P < 0.0005$.

termining platelet sizes and platelet counts on three CH dogs and one normal dog during a 2-week period. The data show highly significant changes with time in platelet sizes for all three CH dogs. Moreover, there was an inverse relation between platelet size and platelet counts, i.e., when platelet counts were normal or below normal, the average platelet sizes were significantly larger than platelets from a normal dog. Conversely, when platelet counts in CH dogs were elevated above normal values, the average platelet sizes were decreased to significantly below the normal control dog values.

Figure 2 shows the results of platelet counts of a CH dog and a normal dog during a 5-day period of time. Also shown are the values for percentage of ^{35}S incorporation into platelets of assay mice after injection of fractions of dog plasma from the two dogs. The platelet count of the CH dog on Day 0 was only slightly above that of the normal dog; platelets of the CH dog then increased at a rapid rate and at 2 days reached two times the level found in the normal dog. The platelet count leveled off by Day 3 and decreased drastically by Day 4. Plasma TSF levels were elevated at the beginning of active thrombopoiesis on Days 0 and 1 ($P < 0.05$ and $P < 0.01$, respectively). However, when the platelet counts were elevated to approximately two times the normal control count, the TSF levels dropped below (not statistically significant at the 0.05 level) the TSF levels of the normal control dog and remained low for another day. When the platelet count dropped to below the normal level (on Day 4), the TSF titer once again increased to slightly above normal levels.

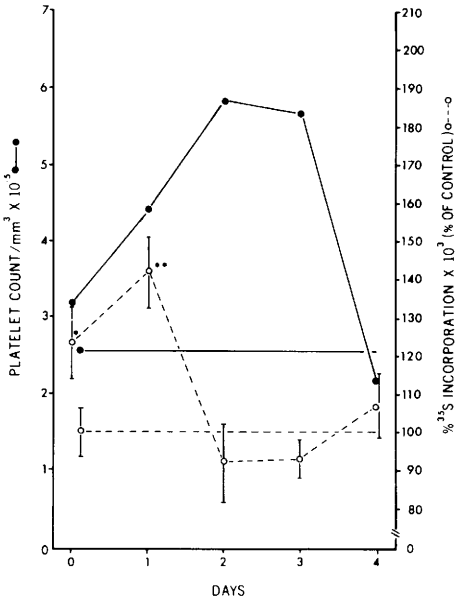

FIG. 2. Platelet counts of a CH and a normal dog, and TSF assay of plasma samples collected daily for 1 week. Percentage of ^{35}S-incorporation values was obtained from five to six mice after injection of 20 mg of plasma extracts at each point; vertical lines represent the standard error. Horizontal solid line represents the platelet count of the normal control dog, and horizontal dashed line represents the percentage of ^{35}S-incorporation of 15 mice injected with a pool of plasma extracts collected daily from a normal dog. Platelet counts of normal dog varied from 2.40 to 2.68 $\times$ $10^5/mm^3$. ^{35}S values were significantly elevated over control values: $^*P < 0.05$; $^{**}P < 0.01$.

Discussion. Cyclic hematopoiesis in dogs has been extensively studied by several investigators (10, 11, 14, 19) and this syndrome in dogs serves as a useful model to compare with a similar disorder in humans, cycling thrombopoiesis (12, 13). Although the cause of the disorder in dogs remains to be determined, it seems clear that platelets cycle from about 125,000 to 900,000/mm^3 and the cycles occur at 11- to 14-day intervals (9, 19–21). In preliminary experiments, several littermate control dogs of this strain had platelet counts of 200,000 to 500,000/mm^3, with a small amount of variation from day to day. Also, no differences in platelet counts were observed in dogs heterozygous for the CH gene when compared to other dogs that were proven to be free of the gene. Platelet sizes and thrombopoietin levels have apparently not been reported previously in CH dogs.

The results of several erythropoietin studies in CH dogs have been presented recently (22–24). Although reticulocytes appear to cycle at regular intervals, erythropoietin could not be detected in dog plasma without stimulation by hypoxia (23) or bleeding (24). Lange and Jones (22) hypothesized that oscillations of erythropoiesis and thrombopoiesis might result from the production of a "poietin-controlling-factor" that stimulates the production of specific factors (i.e., erythropoietin, etc.) leading to in-

creases in reticulocytes, platelets, and monocytes. These authors thought that such a sequence of events would explain the apogee of platelet counts at a time when the nadir of granulocyte counts is reached. They also postulated that feedback mechanisms might be present.

The present work describes an animal model in which average platelet size changes in concert with cycling of platelet production. It should also be mentioned that Booyse *et al.* (25), employing a sucrose density gradient, noted a larger percentage of heavy platelets following recovery from thrombocytopenia in a patient with thrombopoietin deficiency (12). It, therefore, seems possible that CH dogs will be useful for studying the causes of cycling thrombopoiesis disorders in man.

Minter and Ingram (7) have proposed that large-heavy platelets obtained from acutely bled dogs are "stress" platelets and that these platelets do not decrease in size as they age. Other workers (1–6) have presented data that are consistent with a decrease in platelet size with age. In the present work, large platelets were found in unmanipulated dogs when the platelet counts were only slightly below normal. It was thought that blood loss was not a factor responsible for changes in number and size of platelets because the blood sample was small, 3 ml or less per day, which is less than 0.7% of the total blood volume of these dogs. Four to six days after maximum size, the average platelet sizes were significantly smaller than platelet sizes from a normal dog. Dogs are reported to have platelet life spans of about 8 days (26). However, the data of the present report do not conclusively prove that platelets in these dogs decrease in size with age, since platelet life spans were not measured.

It should be mentioned that in the present work, platelet size increased while the platelet counts were decreasing. These results indicate that when platelet counts are low, megakaryocytes are releasing large platelets, and when platelet counts are high, small platelets are either being produced or are found in the circulation as a result of reduced thrombopoiesis, possibly by a result of negative feedback on TSF levels. Further work, therefore, is needed in these dogs to clarify these results. It also seems possible that large platelets produced by stress (such as bleeding) might behave differently from platelets that are normally produced.

Regardless of whether large platelets are normally produced or "stress" produced, they can be conveniently employed to predict megakaryocyte numbers and, hence, platelet production (27). Moreover, it is well known that bleeding releases erythropoietin, and Jackson *et al.* (28) have proposed that erythropoietin might be an inducer of thrombopoiesis. In fact, some experiments (29) have shown that blood loss and/or iron deficiency has an effect on thrombopoiesis. It therefore seems possible that erythropoietin might have, in previous studies, produced "stress" platelets. In the present work, which demonstrated remarkable changes in platelet size without bleeding, erythropoietin was apparently not the cause of increased platelet sizes, but large platelets were probably the result of thrombopoietin action in dogs with cycling thrombopoiesis.

Previously, Weiner and Karpatkin (4), employing a thrombopoietic stimulus (injection of plasma from thrombocytopenic guinea pigs into recipient animals), showed a 2.7-fold increase in numbers of large platelets compared with a 1.5-fold increase in platelet counts after a 4- to 5-day lag period. These experiments were confirmed in rabbits (6). Whereas these workers (4, 6) used exogenous sources of TSF and found changes in platelet sizes, we have shown here both decreased platelet sizes in dogs and increased levels of endogenous TSF at a time when the platelet count was being regenerated (active thrombopoiesis). Moreover, TSF was not detected in the circulation when platelet counts of the dog rose to more than two times the normal count. The data of the present work agree with previous reports indicating that average platelet size increases in animals with TSF-stimulated thrombopoiesis.

Severe cyclic thrombocytopenia has been reported (13) in a young woman. This rare phenomenon is of considerable theoretical interest in relation to platelet kinetics. Plasma TSF levels were measured and the

results suggested that TSF deficiency was the underlying cause of the disease. The data of the present work agree with the finding in the human case of increased TSF at the beginning of active thrombopoiesis. Also, the plasma TSF levels of a CH dog were below normal (but not significantly) when the platelet count was about two times the normal count. Whether TSF deficiency causes platelet cycling in dogs, however, remains to be proven.

Summary. Platelet size, platelet count, and thrombopoietin levels in plasma fractions were determined in cyclic hematopoietic (CH) dogs. Results show that platelet sizes varied inversely with platelet counts of CH dogs; significantly elevated levels of thrombopoietin were found in fractions of plasma from CH dogs at the beginning of active thrombopoiesis. These dogs serve as a useful model for studying cycling thrombopoiesis in man.

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