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Platelet Function Studies in Dogs with Cyclic Hematopoiesis[1] (39538)

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Cyclic neutropenia (CN) or cyclic hematopoiesis (CH) in man is characterized by regular attacks at 21-day intervals of fever, mucosal ulcerations, and neutropenia (1). The duration of the cycle remains constant for most blood elements, but the phase of the cycle or the onset of each element cycling is staggered (2). In 1967 a similar disease was reported in grey collie dogs (3), and the similarity of the disorder in man and dogs has now been cited in numerous reports (4–7). The disease is characterized in dogs as an autosomal recessive trait phenotypically expressed as a grey coat color. Cyclic neutropenia, as well as cycling of the other blood elements, occurs on an 11- to 12-day cycle in the collie and is accompanied by frequent infections during the neutropenic episodes. Since similar cellular fluctuations exist in both humans and dogs, a similar hematopoietic disorder has been hypothesized (8).

Most of the studies concerning cyclic hematopoiesis have dealt with leukocytes and erythrocytes, but not with platelets. Since all the blood cell types may exhibit a cyclic nature, one could hypothesize that blood platelets in CH dogs undergo a cyclic functional alteration with age as has been shown in a patient with thrombopoietin deficiency (9). Therefore, the purpose of this study was to determine if the cyclic nature of the peripherally circulating blood platelets resulted in an effect on platelet adhesion and platelet clot retraction.

Materials and methods. Platelet adhesion. Platelet adhesion or retention index was determined by the Salzman method (10).

Platelet clot retraction. Platelet clot retraction was determined according to the method outlined by McDonald *et al.* (11). Eight to ten milliliters of blood were withdrawn from the jugular vein into syringes containing 0.2 vol of a 1% Na_2-EDTA solution in 0.538% saline. The platelet-rich plasma (PRP) was separated from the other blood cells by centrifugation (200*g*) for 10 min at 5°. A 0.5-ml sample of PRP was removed and the remaining blood was centrifuged at 760*g* for 30 min to obtain the platelet-poor plasma (PPP). A Particle Data Celloscope was used to determine the number of platelets in suspension (1:2000 dilution in Isoton). Dilutions were made of PRP samples to final counts of 300,000, 150,000, 75,000, 37,500, 18,750, and 9,375 platelets/mm^3 in a 0.2-ml sample of Na_2-EDTA-PPP. A 0.5-ml sample of the Na_2-EDTA-PPP containing a 0.2 vol of 0.33 *M* $CaCl_2$ was added to each of the six platelet dilutions for each dog; then bovine thrombin (0.05 ml containing 50 U/ml) was added to initiate clot formation. The final volume of 0.75 ml contained 1.88×10^6 to 60×10^6 platelets, 0.033 mmoles of Ca^{2+}, 1.2 mg of Na_2-EDTA, and 2.5 U of thrombin. The samples were incubated in a 37° water bath for 10 min and the clots were separated from the walls of the tube by use of a small spatula. After a 2 hr incubation, the clots (along with trapped plasma) were weighed to determine the extent of retraction. The data were analyzed by linear regression with the platelet concentrations as the independent variable and the weights of the clots as the dependent variable.

Platelet clot retraction with platelet–plasma combination. In some experiments the plasma was separated from the platelets by centrifugation of the PRP (760*g*) at 5° for 15 min. The plasma from normal, CH, and transplant-CH dogs (CH dogs with normal marrow transplant) was combined with the platelets from normal, CH, and transplant-CH dogs to obtain the following mixtures of

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platelets and plasma: platelets from normal dogs with plasma from either normal, CH, or transplant-CH dogs; platelets from CH dogs with plasma from either normal, CH, or transplant-CH dogs; and platelets from transplant-CH dogs with plasma from either normal, CH, or transplant-CH dogs. Platelet clot retraction was then measured as described above.

Transplant technique. The bone marrow transplants were carried out as previously described (12, 13). Prior to transplantation the dogs used in these studies (normal dog No. 220 bearing a CH marrow graft, CH 309, and CH 310 bearing a normal marrow graft) were exposed to a lethal dose of irradiation (1250 R) over a 2-hr period.

Neutrophil staining and counting. The neutrophil counts were done by standard techniques as previously described (14). The data were expressed as the absolute neutrophil count, i.e., the percentage of neutrophils multiplied by the total leukocyte count.

Fibrinogen determinations. Fibrinogen determinations were made by use of a commercially available kit (Data-F Fibrinogen Determination, DADE Division, American Hospital Supply Corporation, Miami, Fla.). Bovine thrombin was added to a 1:10 dilution of fresh citrated plasma (diluted with Owren's Veronal buffer) and the clotting time was compared to that of a standardized fibrinogen preparation.

Results. Figure 1 illustrates the daily neutrophil counts of CH and normal dogs. The absolute neutrophil count for normal dogs varied from 5.3 to 7.3 $\times$ $10^3/mm^3$, whereas the neutrophil counts in the CH dogs varied from 0.15 $\times$ $10^3/mm^3$ during neutropenia (Days 7–8) to 17.3 $\times$ $10^3/mm^3$ immediately after neutropenia. Generally, recovery from the neutropenia was accompanied by a severe leukocytosis only when infections were present.

Figure 2 shows daily platelet counts for CH dogs and the average platelet count for normal littermates. The platelets in CH dogs reach a peak immediately before neutropenia. The platelet cycle is, therefore, out of phase with the neutrophil cycle.

As shown in Fig. 3, the percentage of platelet adhesion was consistently depressed in CH dogs as compared to normal controls. The percentage of adhesion of platelets from CH dogs remained below 32% during the entire neutrophil cycle. The results of

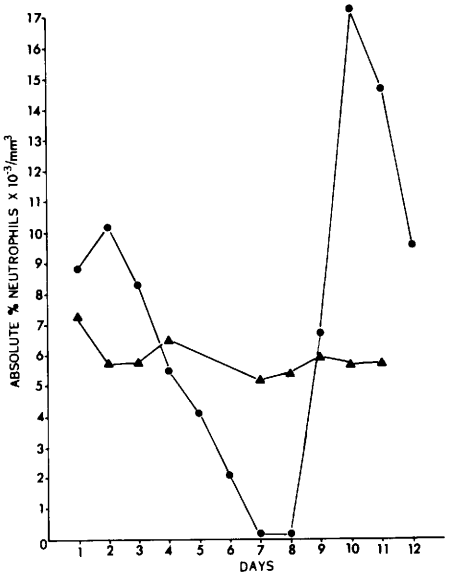

FIG. 1. Absolute percentages of neutrophil counts of normal (No. 358 and 360, ▲) and CH dogs (No. 346 and 181, ●) during one complete cycle.

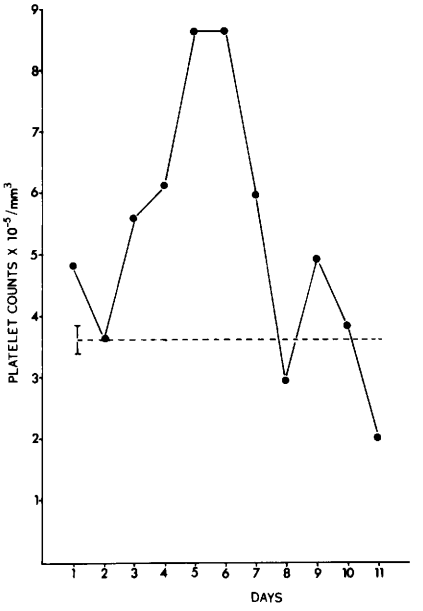

FIG. 2. Peripheral platelet counts of normal (No. 358 and 360, shown by horizontal line) and CH dogs (No. 346 and 181, ●) during one complete cycle.

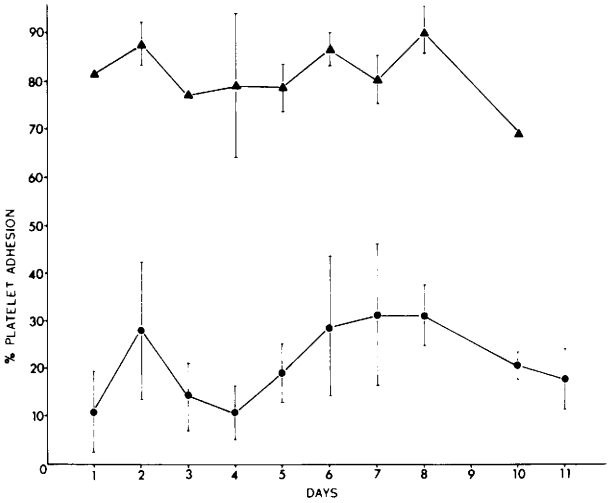

FIG. 3. Adhesion of platelets from normal (No. 359 and 360, ▲) and platelets from CH dogs (No. 361, 362, and 364, ●) during one complete cycle.

testing the percentage of adhesion of platelets from a CH dog and three dogs transplanted with bone marrow are shown in Table I. The platelet adhesiveness index was depressed to below normal (44%) in a normal dog transplanted with marrow from a CH dog. Conversely, the platelet adhesion values were normal (80–90%) in both CH dogs after transplantation with normal marrow. The CH dog without transplantation showed a low platelet adhesion in agreement with data for other dogs presented in Fig. 3.

Table II shows the least-squares analysis of the platelet clot retraction data obtained from normal and CH dogs over a 12-day period. The clot retraction of platelets from CH dogs was consistently less than that of platelets from normal dogs (the larger the intercept the larger the clot and the less functional are the platelets). As in the platelet adhesiveness studies, there was no relationship between platelet clot retraction data and the cyclic fluctuations of the platelets or neutrophils. The intercepts of the clot retraction were significantly ($P < 0.025$) different from one another, whereas the slopes were not.

Table III shows the results obtained from clot retraction studies after platelet-plasma combinations involving platelets and plasma from normal and CH dogs and a CH dog transplanted with normal marrow (trans-

TABLE I. EFFECT OF BONE MARROW TRANSPLANTATION ON PLATELET ADHESION.

Treatment	Adhesion (%)
Normal dog No. 220 transplanted with CH marrow	44
CH dog No. 310 transplanted with normal marrow	80
CH dog No. 309 transplanted with normal marrow	90
CH dog No. 338 (adult), no transplant	24

plant-CH). When platelets were incubated in normal plasma (a–c) there was no significant change in the intercept among the three groups (a–c). However, when one compares the intercepts obtained after incubation of platelets in normal plasma (a–c) with those in CH plasma (d–f), there is a significant ($P < 0.05$) increase in the values of the intercepts indicating less clot retraction or less functional platelets. When platelets were incubated with plasma from a transplant-CH dog (g–i), the intercepts were consistently decreased indicating an increased functional capacity perhaps due to the presence or absence of a factor in the transplant-CH dog's plasma.

Since the plasma from either normal or transplant-CH dogs corrected the clot retraction defect, the existence of an altered plasma factor was considered. Since fibrinogen levels are known to influence platelet

TABLE II. CLOT RETRACTION OF PLATELETS FROM NORMAL AND CH DOGS.

Treatment[a]	Number of dogs	Number of determinations	Least-squares analysis: Intercept ± SE (weight of clot (mg))	Least-squares analysis: Slope ± SE (platelets × 10^{-5}/mm^3)
Normal	2	72	2.27 ± 0.31	−5.91 ± 0.93
CH	2	90	3.36 ± 0.40[b]	−8.23 ± 1.11

[a] The dogs used in this study were as follows: two normal dogs No. 358 and 360 (9–11 months old); two CH dogs No. 181 (2½ years old) and No. 346 (10 months old).

[b] Platelet clots from CH dogs were significantly larger than clots formed by platelets of normal dogs ($P < 0.025$).

TABLE III. CLOT RETRACTION EXPERIMENTS WITH VARIOUS PLATELET-PLASMA CONCENTRATIONS.

Treatment[a]: Platelets	Treatment[a]: Plasma	Number of determinations	Least-squares analysis: Intercept ± SE (weight of clot (mg))	Least-squares analysis: Slope ± SE (platelets × 10^{-5}/mm^3)
a. Normal	Normal	24	1.44 ± 0.28	−4.62 ± 3.11
b. CH	Normal	24	1.50 ± 0.43	−2.40 ± 0.09
c. Transplant-CH	Normal	12	2.02 ± 0.10	−6.49 ± 0.17
d. Normal	CH	24	3.00 ± 0.86	−4.54 ± 10.72
e. CH	CH	12	3.29 ± 1.07	−2.92 ± 0.53
f. Transplant-CH	CH	12	3.15 ± 1.09	−6.07 ± 0.51
g. Normal	Transplant-CH	12	0.52 ± 0.00	−1.37 ± 0.00
h. CH	Transplant-CH	12	0.64 ± 0.01	−1.80 ± 0.43
i. Transplant-CH	Transplant-CH	12	0.69 ± 0.18	−1.58 ± 2.53

[a] The dogs used in this study were as follows: two normal dogs No. 358 and 360 (9–11 months old); two CH dogs No. 181 (2½ years old) and No. 346 (10 months old); and No. 310, a CH dog that had received normal bone marrow 6–8 months prior to experimentation.

function, the concentration of this procoagulant protein was measured during one complete neutrophil cycle. Figure 4 shows the results of the fibrinogen determination on three dogs along with the absolute percentage neutrophil count. The mean values for the normal and transplant-CH dog are depicted for the purpose of illustration rather than the individual counts. In general, fibrinogen levels fluctuate in unison with neutrophil counts during the CH cycle. However, the fibrinogen values for the CH dog are consistently elevated above normal and transplant-CH levels.

Discussion. The results of this study indicate that platelet adhesion and platelet clot retraction were consistently depressed in dogs with cyclic hematopoiesis (CH) and in a single normal dog transplanted with CH marrow, whereas fibrinogen levels were elevated in the CH dogs. These decreased platelet functions and elevated fibrinogen values were returned to normal levels after homologous bone marrow transplantation from normal donors.

TABLE IV. PLASMA FIBRINOGEN DETERMINATIONS FROM A NORMAL, CH, AND TRANSPLANTED DOGS.

Dog (No.)	Number of determinations	Fibrinogen (mg/dl ± SE)
Normal (360)	6	208.0 ± 16.5
CH (346)	6	442.5 ± 42.7[a]
Transplant[b] (310)	6	242.5 ± 10.1

[a] Plasma fibrinogen levels were significantly higher than normal or transplant ($P < 0.001$).

[b] CH dog transplanted with normal marrow.

There is no apparent explanation for the depressed platelet adhesiveness index in conjunction with the elevated levels of fibrinogen observed in the CH dogs. It does seem possible that the excess fibrinogen "coats" the platelet membrane, thereby covering up the active sites on the membrane which are necessary for adhesion to occur. Mason *et al.* (15) reported that platelet adhesion increases with increasing levels of fibrinogen plateauing after a certain concentration, which would appear contradictory to our findings. One possible explana-

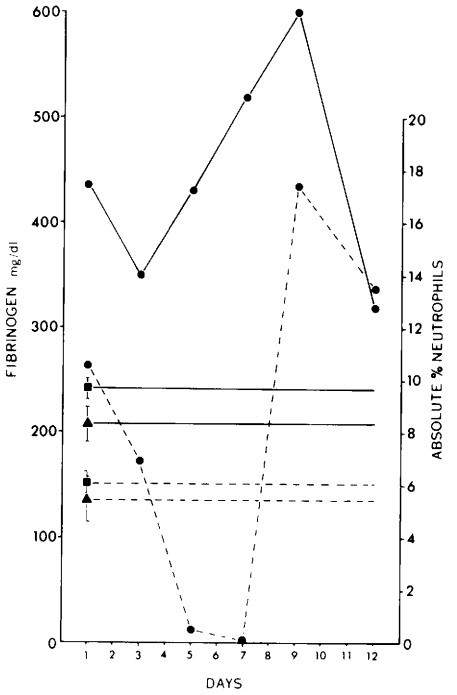

FIG. 4. Fibrinogen levels and absolute percentages of neutrophil counts of a normal (No. 358, ▲), a CH (No. 346, ●), and a transplant-CH (No. 310, ■) dog during one cycle. The fibrinogen and neutrophil values were presented as the mean ± SE for illustrative purposes for the normal and transplant-CH dogs.

tion for the dichotomy could be an altered functional capability of the fibrinogen molecule in the CH dog, or perhaps the platelets themselves are congenitally deficient in CH animals. Another alternative hypothesis could be that platelet function and fibrinogen levels are not causally related to each other, though both are altered by the primary CH process. This seems to be the case in dogs affected by CH disease, especially in view of the findings expressed in Fig. 4.

Although the level of platelet adhesiveness is greatly reduced (Fig. 3), there does appear to be a slight increase in the percentage of adhesion on Days 5–8 following the phase of platelet production on Days 2–6 (Fig. 2). Since these dogs do not normally hemorrhage to death, platelets from CH dogs may be able to respond enough *in vivo* to maintain hemostasis. Perhaps the elevated levels of fibrinogen aid the platelets in their normal hemostatic role in CH dogs.

Depressed platelet clot retraction was also demonstrated in the CH dog. As with the platelet adhesiveness index, the ability of platelets to undergo normal clot retraction was restored after irradiation and reconstitution of CH dogs with normal bone marrow. In fact, the degree of clot retraction in dogs after transplantation was greater than in normal dogs.

Evidence for the existence of a plasma factor's influence on platelet clot retraction was found in platelet–plasma mixture experiments (Table III). Incubation of platelets from CH dogs in plasma from either normal or transplant-CH dogs resulted in normal clot retraction. Conversely, incubation of platelets from normal or transplant-CH dogs with plasma from CH dogs caused a decreased clot retraction.

These facts suggest that some plasma factor(s) is altered in the transplant recipient which enables the platelet function to return to normal limits. A previous study has shown that clot retraction can be altered by some plasma factor in rats (11). Rather than a lack of some plasma factor in the CH dog, an overabundance of a plasma component seemed to be responsible for the altered platelet function. Since plasma fibrinogen levels in CH dogs were consistently elevated over normal or transplant-CH levels, the hypothesis that fibrinogen is the contributory factor is conceivable. It should also be mentioned that fibrinogen levels are increased in dogs with infections such as lymphosarcoma, distemper, mild liver disease, and kidney disease (16). Therefore, the inflammatory changes that accompany CH of dogs (6) may account for the increased levels of fibrinogen. Reconstitution of CH dogs with normal marrow alleviates CH disease and infective processes and, thus, could account for the fibrinogen level's return to normal along with normal platelet adhesion and clot retraction.

Summary. Platelet adhesiveness, platelet clot retraction and fibrinogen determinations were made on dogs with cyclic hematopoiesis (CH), dogs cured of CH disease via bone marrow transplantation, and normal dogs. Platelet adhesiveness and clot retraction values in CH dogs were below normal values throughout the cycle and were not

influenced by neutropenic episodes. However, these platelet functions were normal in CH dogs transplanted with normal marrow. Fibrinogen values of CH dogs did fluctuate with the neutrophil cycle, but were continually elevated when compared to either normal dogs or CH dogs transplanted with normal marrow. By use of platelets and plasma from normal, CH, and transplant-CH dogs, it was shown that plasma from either normal dogs or CH dogs transplanted with normal marrow corrected the CH dogs' platelet clot retraction defect. These studies indicate that a plasma factor is involved in the decreased platelet function of CH dogs; furthermore, the plasma factor may be fibrinogen.

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