To the Graduate Council:

I am submitting herewith a thesis written by Charlotte M. Boney entitled "Platelet Aggregation Studies in Canine Cyclic Hematopoiesis." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for a Master of Science, with a major in Comparative and Experimental Medicine.

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We have read this thesis
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PLATELET AGGREGATION STUDIES IN CANINE CYCLIC HEMATOPOIESIS

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee

Charlotte M. Boney

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ABSTRACT

Investigation of platelet function in dogs with cyclic hematopoiesis (CH) revealed a platelet aggregation disorder. Collagen-induced aggregation of CH dog platelets was significantly below normal, although normal aggregation in response to ADP was observed. The failure to aggregate in response to collagen was not influenced by platelet size. Aggregation was particularly low on days 2, 3, 4 and 14 of the 14-day neutrophil cycle which is typical of CH dogs. The lack of response to collagen suggests a defect in the arachidonic acid pathway of platelet metabolism since platelet-generated thromboxane B_2 was significantly (~70%) below control levels. Platelets from dogs heterozygous for CH demonstrated moderately depressed responses to collagen which were intermediate between the values found for CH dog platelets and normal, mixed breed dog platelets. Not only does this work indicate a platelet defect in CH dogs, but this phenomenon may be useful as a genetic marker in identification of dogs heterozygous for the CH gene.

TABLE OF CONTENTS

CHAPTER	PAGE
I. INTRODUCTION	1
II. MATERIALS AND METHODS	3
Subjects and Reagents	3
Platelet Aggregation Studies	3
Platelet Sizing Studies	4
Thromboxane B_2 Radioimmunoassay	5
Statistical Analysis	5
III. RESULTS	6
IV. DISCUSSION OF RESULTS	15
LIST OF REFERENCES	20
VITA	24

LIST OF FIGURES

FIGURE		PAGE
1	Daily neutrophil counts of a CH dog (●) and a normal dog (o) through one cycle.	7
2	Tracings of typical platelet aggregation patterns in response to ADP (20 μM) and collagen (125 μ g/ml) from a normal dog (NM), a heterozygous dog (HZ) and a CH dog (CH).	8
3	Percent platelet aggregation at 10 minutes after addition of collagen (125 μg/ml) from five CH dogs through one cycle.	10
4	Platelet sizes of a CH dog (●) and a normal dog (o) measured during one cycle.	11
5	The biosynthetic pathway of thromboxane B_2 in platelets. .	17

CHAPTER I

INTRODUCTION

Canine cyclic hematopoiesis (CH) is an autosomal recessive disorder that has been the object of investigation in several laboratories (1-3) since the syndrome was first described in 1967 (2). Inheritance of CH is accompanied by a characteristic grey coat color (4,5). The disease is characterized by neutropenia every 13-15 days in addition to monocytosis, moderate reticulocytosis and a variable thrombocytosis (5-9). The first neutropenic episode usually occurs within the first 12 days of life (3). CH dogs are afflicted with periodic lameness, fever, depression, infections, painful joints and mucosal ulcerations throughout their shortened life spans.

Transplantation of marrow from normal littermates into supralethally irradiated CH dogs resulted in abatement of neutrophil, platelet and reticulocyte fluctuations. Likewise, CH marrow transplanted into normal recipients transferred characteristic manifestations of CH to the recipient dogs (10). This evidence, in addition to earlier studies (11,12) involving CH transplantation, certainly suggests a stem cell defect and establishes the marrow compartment as the primary site of the disease.

Previous studies of CH dog platelets indicated that platelet sizes varied inversely with platelet counts (13). Platelet function tests revealed that platelet adhesiveness and clot retraction values were below normal throughout the cycle (14). These platelet disorders were unaffected by neutropenic episodes and did not cycle. Abnormal adhesiveness and clot retraction indicated the need for studies of the aggregation responses of CH dog platelets. In the present study, further investigation of platelet function in canine cyclic

hematopoiesis has demonstrated the failure of CH dog platelets to aggregate in response to collagen, although normal aggregation with adenosine diphosphate (ADP) was observed. Collagen-induced aggregation of platelets from dogs heterozygous for CH was intermediate between the aggregation response of normal and CH platelets.

CHAPTER II

MATERIALS AND METHODS

Subjects and Reagents

Male and female cyclic hematopoietic dogs of the collie-beagle genotype, all members of the colony maintained by this laboratory (8), were used in these studies. Dogs genetically heterozygous for CH which are breeding members of the colony were used as heterozygous subjects. Normal, mixed breed dogs obtained from municipal pounds, immunized against distemper and treated with parasiticides prior to these studies were used as normal controls. Neutrophil counts were determined daily on CH dogs using a venous blood sample collected into Monoject EDTA vacutainers from Sherwood Medical, St. Louis, Missouri.

Adenosine diphosphate (ADP) and acetylsalicylic acid (aspirin) were purchased from Sigma Chemical Corporation, St. Louis, Missouri. Soluble calf-skin collagen was purchased from Worthington, Freehold, New Jersey. Trisodium citrate was from Fisher, Fairlawn, New Jersey. Isoton was from American Chemical Company, McGaw Park, Illinois. Thromboxane B_2 radioimmunoassay materials were purchased as a kit from New England Nuclear, Boston, Massachusetts.

Platelet Aggregation Studies

These were done according to the method of McDonald and Clift (15) using a Chrono-log aggregometer (Broomall, Pa.) with a model 702 strip chart recorder. Blood was drawn from the jugular vein each day between 8 and 9 A.M. A standard volume of nine ml blood was mixed with 136 mM trisodium citrate in a blood to buffer ratio of 9:1 in a sterile plastic tube. Blood

samples were diluted with saline (5:3, v/v) prior to centrifugation at 160 g for 10 minutes at 22°C. Three ml of the resulting platelet-rich plasma (PRP) were removed to plastic tubes and the blood samples subsequently centrifuged at 850 g for 10 minutes at 22°C to obtain platelet-poor plasma (PPP). Platelet-rich plasma was diluted with PPP to a concentration of 100,000-250,000 platelets/ μl prior to aggregation studies.

The aggregometer base line was set at 10% light transmission using PRP. Full transmission was set at 90% light transmission using PPP. Platelet suspensions in a volume of 0.45 or 0.48 ml were maintained at 37°C and stirred at a constant rate (1,200 rpm) while in the aggregometer. Aggregating materials used were 20 μ l ADP and 50 μ l collagen. ADP was diluted to 250 μM in saline, kept frozen, and portions thawed prior to use. Collagen was diluted daily to 1.25 mg/ml in modified Tyrodes buffer (0.137 M NaCl, 0.003 M KCl, 5.5 mM glucose, 0.012 M $NaHCO_3$, 0.3 mM NaH_2PO_4, pH 7.35) and kept at 4°C. The maximum percent transmission at 3 minutes after addition of ADP and at 10 minutes after addition of collagen was measured. The percent platelet aggregation was calculated as follows:

$$\% \text{ aggregation} = \frac{90 - PRP}{80} \times 100.$$

Platelet Sizing Studies

Average platelet size (on a logarithmic scale) for each subject was determined by use of an Electrozone/Celloscope (Particle Data, Inc., Elmhurst, IL) as previously described (16). All sizing of platelets was performed at 22°C with a 48- μ m diameter orifice. Platelet-rich plasma samples were diluted 1:2000 (5 μ l PRP and 10 ml Isoton) yielding a platelet concentration

of 0.5-2.0 x 10^4 platelets/100 μl in order to maintain a coincidence error of less than 1%. The instrument settings were: log 10 (logarithmic span of ~ 10 doublings of particle volume or 10:1 in diameter), current 6.5, and gain 3. The discrimination range was set at 40-900. Analysis involved a particle size range of 1.30 to 55.0 μm^3 volume. Calibration was maintained constantly by using latex particles of known size and frequent checking.

Thromboxane B_2 Radioimmunoassay

Thromboxane B_2 (TXB_2) was determined by radioimmunoassay using a kit from New England Nuclear. The sensitivity of the radioimmunoassay was approximately 0.8 picograms TXB_2 with less than 3.9% cross-reactivity for PGD_2 and less than 0.23% cross-reactivity for all other prostaglandin compounds. Following aggregation of 0.5 ml PRP with 125 μg/ml collagen at 37°C for 10 minutes, 0.5 ml of 20 μg/ml aspirin in phosphate-buffered saline was added to inhibit further TXB_2 production. Samples were centrifuged immediately at 850 g for 10 minutes. A portion of the resulting supernatant from platelet aggregates was stored at -76°C or assayed immediately. In addition, 0.2 ml of autologous PPP was diluted with 0.2 ml of 20 μg/ml aspirin and either frozen or assayed immediately as above. Samples were diluted 10-fold with buffer for assay of plasma and platelet-released TXB_2.

Statistical Analysis

The Student's *t* test was used to determine statistical significance.

CHAPTER III

RESULTS

Daily neutrophil counts of a CH and normal dog are illustrated in Figure 1. The cycling of neutrophils of the CH dog is a typical manifestation of cyclic hematopoiesis demonstrated by earlier studies (6-8,14). Previous reports from our laboratory (8,14) demonstrated that peripheral platelet counts in CH dogs cycle out of phase with the neutrophils and that platelet counts vary inversely with platelet size (13).

Figure 2 shows no difference between a normal, heterozygous or a CH dog (cycle day 4) in platelet aggregation following addition of ADP. Platelet aggregation response to ADP was consistently normal in all subjects. There was no delay time and the slope was steep ($\simeq 75^{o}$), indicating a rapid change in light transmission due to aggregation. However, profound differences between dogs of different genotypes were seen in both maximum aggregation and slope value after addition of collagen. The results graphed in Figure 2 are typical of aggregation responses observed in all normal, heterozygous and CH subjects. Normal dog platelets generally present a delay time of 4 to 5 minutes after addition of collagen followed by a steep slope ($\simeq 64^{o}$). Heterozygous dog platelets also have a delay time of 4 to 5 minutes prior to aggregation, but the slope ($\simeq 40^{o}$) and the maximum aggregation is not as great as in normal dog platelets. Platelets from CH dogs fail to aggregate in response to collagen, therefore there is no measureable slope value or true delay time. The only similarity in all subjects' aggregation patterns is the positive shape change of platelets, indicating receptor-ligand binding, which can be seen immediately following addition of collagen.

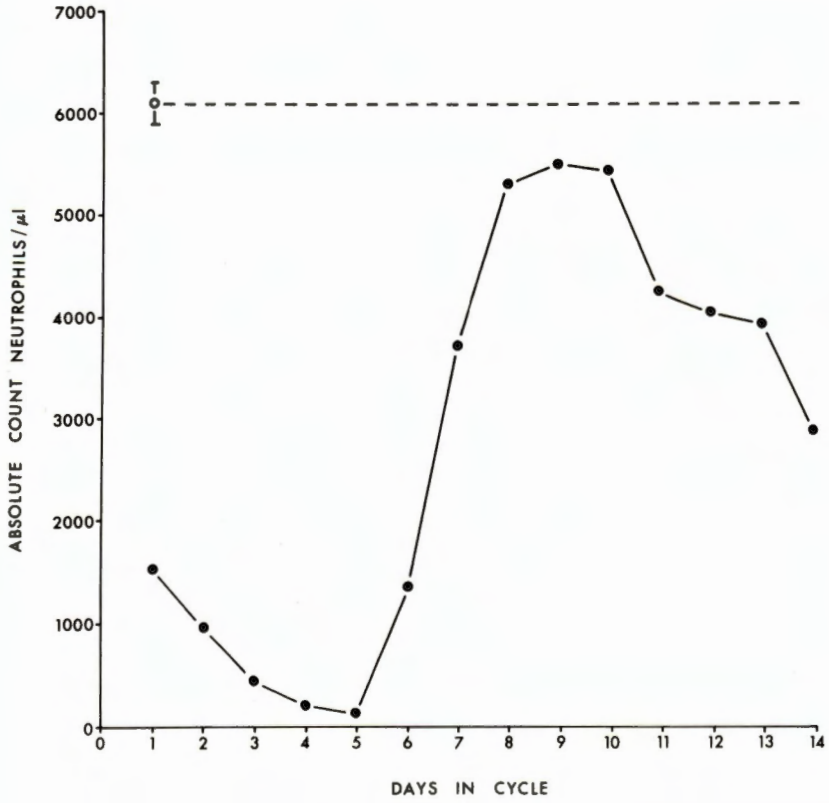

Figure 1. Daily neutrophil counts of a CH dog (●) and a normal dog (o) through one cycle. Day one of the cycle is defined as the first day the peripheral count falls below 1600 neutrophils/µl. The normal count is the mean ± SE for 8 determinations. CH dogs generally reach normal levels on days 6-12.

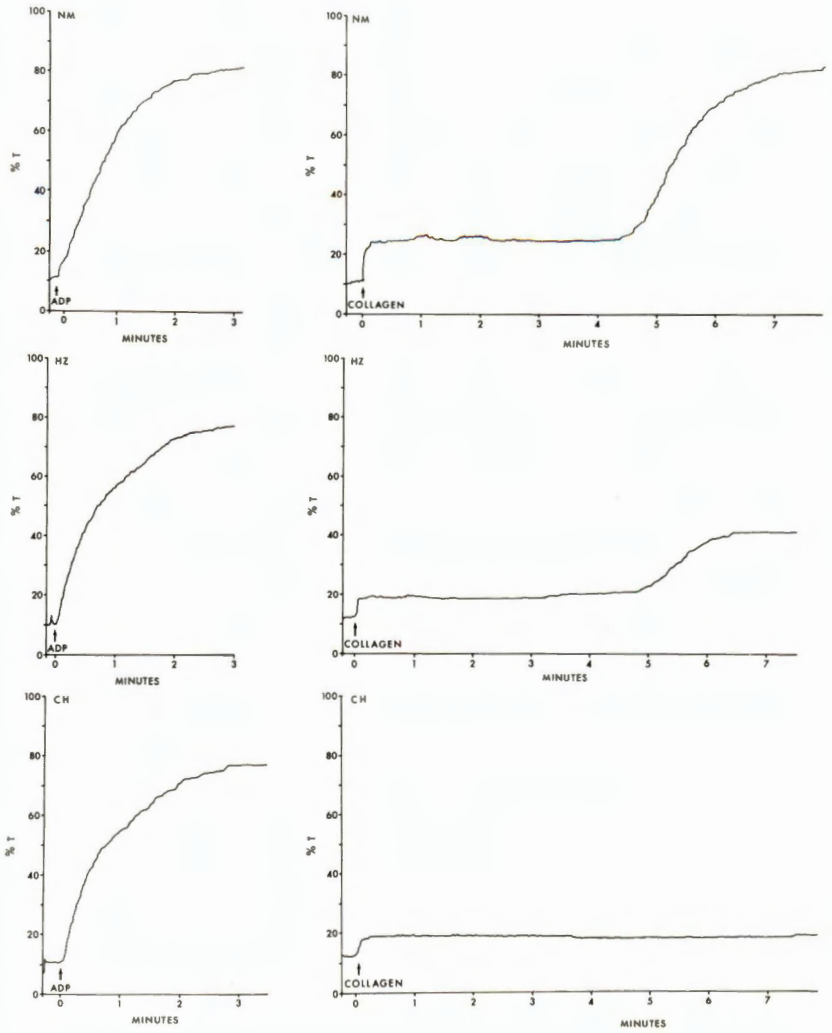

Figure 2. Tracings of typical platelet aggregation patterns in response to ADP (20 μM) and collagen (125 μg/ml) from a normal dog (NM), a heterozygous dog (HZ) and a CH dog (CH). %T is percent light transmission. Irregularities in the early portion of the curve indicate shape change of platelets.

Due to the cycling nature of peripheral platelet counts, collagen-induced aggregation of platelets from CH dogs was studied throughout a cycle. As shown in Figure 3, aggregation response may cycle since the response to collagen on days 2, 3, 4 and 14 was lower ($P \leq 0.05$) than the peak on day 8. However, CH platelet aggregation did not appear to be affected by neutropenic episodes since significant differences were not found between cycle days 1,2,3 and 10,11,12. In addition, no relationship was found between platelet aggregation and platelet size (Figure 4). Although peripheral platelet counts from CH dogs were found to cycle from normal to above normal values as reported earlier (8), we found no effect of platelet count on aggregation response (data not shown).

In order to make a more extensive comparison of platelet aggregation among normal, heterozygous and CH dogs, collagen and ADP determinations were made and pooled for each subject type and are shown in Table 1. No defect in ADP response was present in either heterozygous or CH dogs. However, there was a significant difference ($P < 0.0005$) in the response of platelets to collagen from both heterozygous and CH dogs compared to normal dogs. The disorder was particularly profound in CH platelets.

Thromboxane B_2 (TXB_2) is the inactive hydration product of thromboxane A_2 (TXA_2), the major arachidonic acid metabolite in platelets which mediates the aggregation response to thrombin and collagen (17-19). Since CH dog platelets failed to respond to collagen and heterozygous dog platelets responded poorly, immunoreactive TXB_2 formation by platelets was measured. These results are shown in Table 2. Platelets from both heterozygous and CH dogs produced less TXB_2 than normal dogs. Moreover, platelets from CH dogs produced less TXB_2 than heterozygous dog platelets. TXB_2 was determined

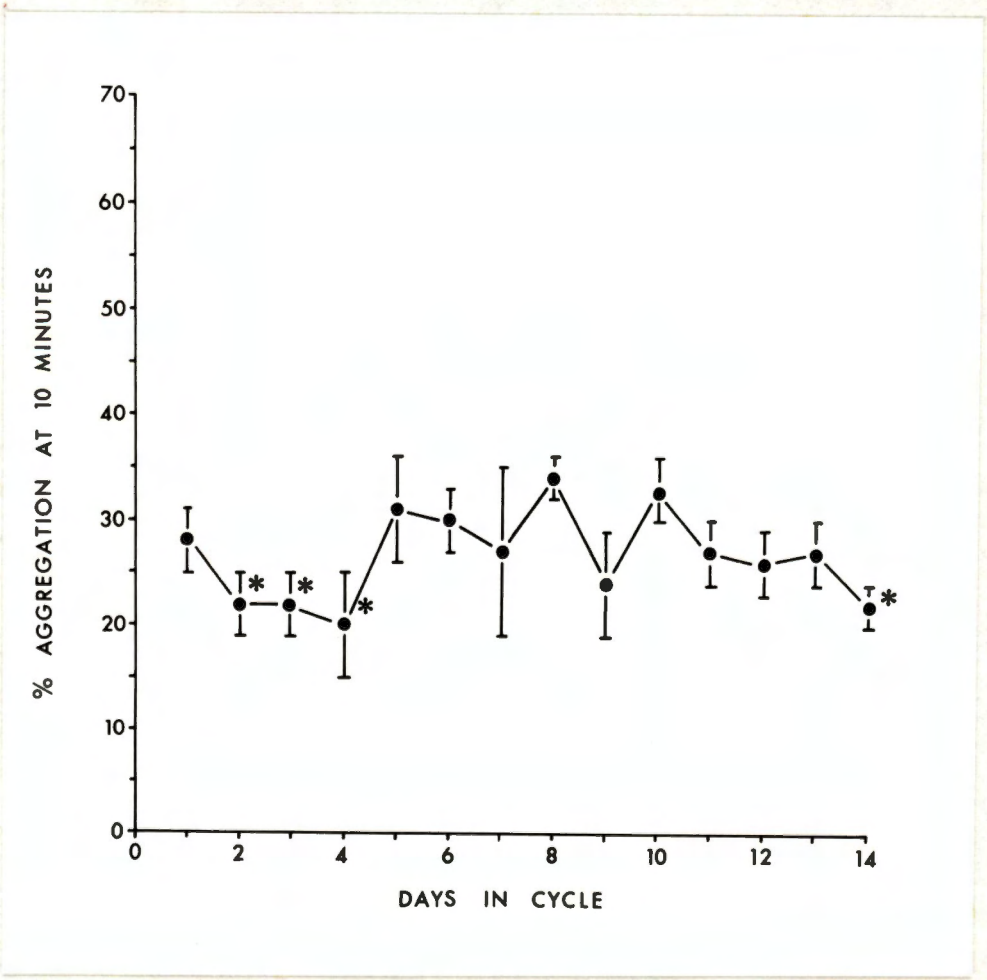

Figure 3. Percent platelet aggregation at 10 minutes after addition of collagen (125 μg/ml) from five CH dogs through one cycle. Percent aggregation is presented as the mean ± SE. Percent aggregation on days 2, 3, 4 and 14 was significantly less ($P \leq 0.05$) than on day 8.

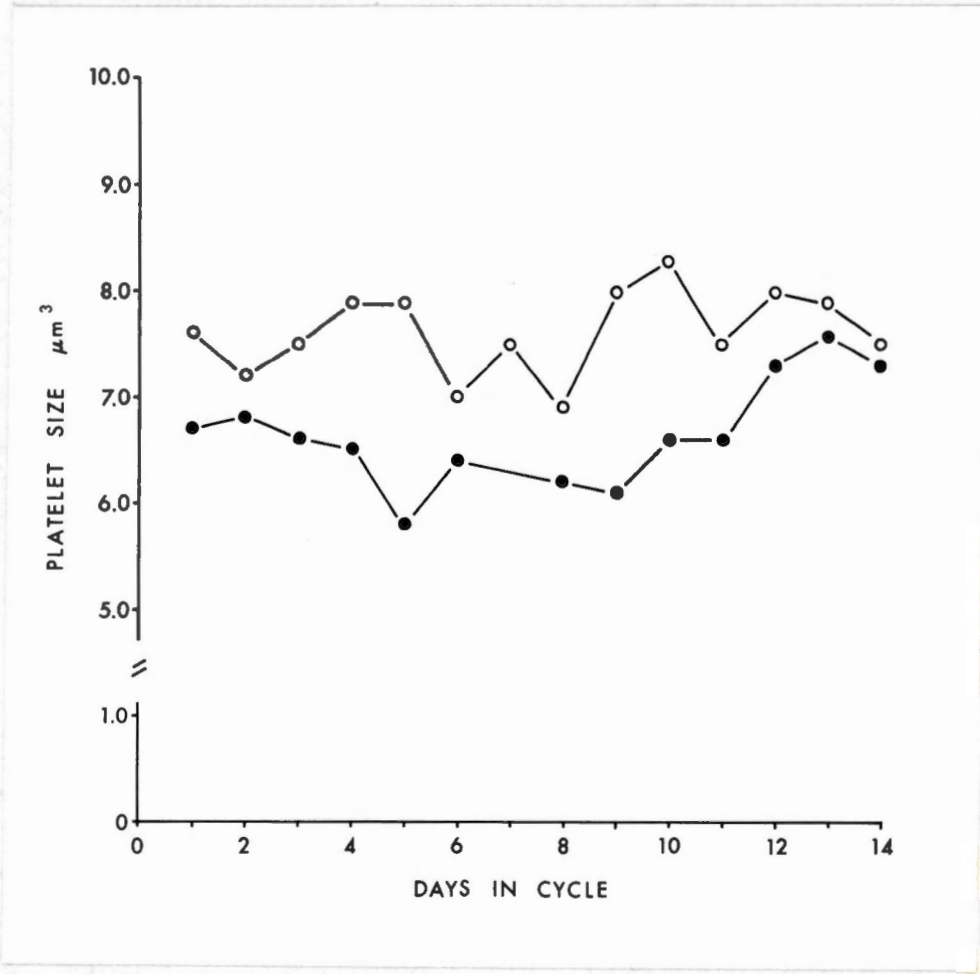

Figure 4. Platelet sizes of a CH dog (●) and a normal dog (o) measured during one cycle. Sizes presented are the arithmetic mean size of a normal distribution. CH dog platelets were not found to be significantly smaller than normal.

TABLE 1. AGGREGATION OF PLATELETS FROM NORMAL, HETEROZYGOUS AND CH DOGS

Subject	Number of dogs	Number of determinations	20μM ADP		125 μg/ml collagen	
			%	slope	%	slope
Normal	6	23	85.6±1.4	72°±1	88.0±1.6	64°±1
Heterozygous	12	18	83.4±1.6	72°±1	54.6±4.2[a]	47°±3[a]
CH	5	34	85.9±1.6	76°±1	26.3±1.4[b]	4°±1[b]

Percent aggregation and slope of response were pooled and are presented as the mean ± SE.

[a]Percent aggregation and slope after addition of collagen were significantly less than normal subjects ($P < 0.0005$).

[b]Percent aggregation and slope after addition of collagen were significantly less than normal subjects ($P < 0.0005$) and significantly less than heterozygous subjects ($P < 0.0005$).

TABLE 2. COLLAGEN-INDUCED PLATELET PRODUCTION OF THROMBOXANE B_2 IN NORMAL, HETEROZYGOUS AND CH DOGS

Subject	Number of dogs	Number of determinations	% of control TXB_2[a]
Normal	4	9	100.4 ± 2.7
Heterozygous	9	11	64.5 ± 4.1[b]
CH	4	15	30.3 ± 3.0[c]

[a]Platelet-generated TXB_2 is expressed as a percentage of normal control values. Percentages of control from all radioimmunoassays were compiled and are presented as the mean ± SE.

[b]TXB_2 values were significantly less than normal ($P < 0.0005$).

[c]TXB_2 values were significantly less than normal ($P < 0.0005$) and significantly less than heterozygous subjects ($P < 0.0005$).

in both PPP and collagen-stimulated PRP. Because of extraplatelet sources of TXB_2 in blood, the amount of TXB_2 in PPP was subtracted from the amount of TXB_2 in PRP and divided by the number of platelets in the sample. In addition, factors such as bleeding of subjects, time between isolation of PRP and aggregation with collagen, assay of fresh versus frozen samples, and interference of immunoprecipitation by plasma components may affect measurable TXB_2 levels; therefore, data are presented as percent of control.

CHAPTER IV

DISCUSSION OF RESULTS

The present investigation has explored the aberration of platelet function in dogs with CH. We have found that collagen failed to induce aggregation in CH dog platelets while normal aggregation with ADP was observed. We suggest that this abnormality involves endogenous platelet function and that it is not a defect in collagen binding to its receptor. After the addition and subsequent binding of ADP or collagen to its receptor, platelets become rounded and more symmetrical immediately before they become sticky (20). This effect on platelet shape was seen in the aggregometer tracings immediately after addition of ADP and collagen, as well as under the phase-contrast microscope.

The platelet malfunction in CH is not consistent with most of the congenital abnormalities of platelet function which involve decreased responsiveness to aggregating agents (21). ADP-induced aggregation is normal and shape change does occur in CH dog platelets. In Glanzmann's Thrombasthenia, platelets undergo shape change but the primary aggregation reaction to ADP does not occur. Adherence and the platelet release reaction (specific secretion of granule-stored material) are normal, but no aggregation in response to high levels of ADP, collagen or thrombin is seen. The defect is thought to be in platelet surface glycoproteins (22). The Bernard-Soulier or giant platelet syndrome is characterized by normal aggregation to ADP, collagen, and thrombin but no shape change occurs and adhesion to the subendothelium is defective (22).

There are two main types of platelet release abnormalities: storage pool disease and an "aspirin-like" defect (22). In storage pool disease, the primary

phase of ADP-induced aggregation is normal (reversible response without release of granule contents), but second phase aggregation (biphasic irreversible aggregation due to the release reaction) with high concentrations of ADP is absent and the response to collagen is impaired. The defect is believed to be due to a low number of dense bodies with decreased concentrations of granule contents (ADP, ATP, serotonin, Ca^{++}) (21). In the present study, high concentrations of ADP (20 μM) were used and the aggregation response was maximal. When concentrations between 2 μM and 10 μM were used, reversible aggregation was observed in all cases (data not shown). Therefore, we conclude that storage pool disease is not responsible for the platelet abnormality of CH dogs.

The "aspirin-like" defect is so named because its hematologic symptoms parallel those produced by aspirin and other non-steroidal anti-inflammatory drugs. Platelet granule numbers and contents are normal and aggregation with ADP is normal. However, the response to collagen, which induces the release reaction and stimulates endogenous prostaglandin biosynthesis from arachidonic acid by activation of phospholipase A_2, is impaired (19). The significance of prostaglandins, particularly thromboxane A_2 (TXA_2), in platelet aggregation is now well established (23,24). Inhibition at any point in the arachidonic acid pathway (Figure 5) results in poor aggregation due to insufficient production of TXA_2 (19). Aspirin irreversibly acetylates cyclo-oxygenase and thereby prevents TXA_2 production (25,26). Agents that inhibit the action of phospholipase A_2 (19) or thromboxane synthetase (27) also block the synthesis of TXA_2.

The aggregation profile of CH dogs suggests a defect in platelet arachidonic acid metabolism, particularly since TXB2 production was low.

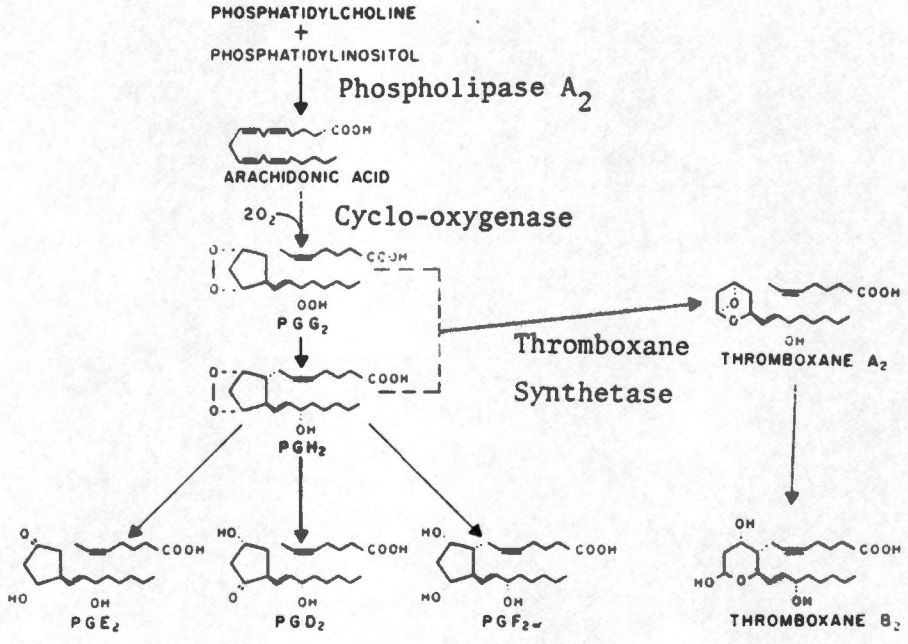

Figure 5. The biosynthetic pathway of thromboxane B_2 in platelets.

Several laboratories have used TXB_2 radioimmunoassays to correlate abnormal platelet function with impaired thromboxane synthesis (26,28,29). In addition, a deficiency of platelet cyclo-oxygenase has been reported (30) in a human patient having an aggregation profile almost identical to that of CH dogs. Platelets that are indeed defective in the arachidonic acid pathway fail to aggregate in response to arachidonic acid. We did not test the CH dog platelet response to arachidonic acid since, unlike human platelets, dog platelets usually fail to aggregate in vitro in response to arachidonic acid and the addition of epinephrine does not uniformly convert arachidonic acid-insensitive platelets to arachidonic acid-sensitive platelets (31).

It is now well established that high intracellular concentrations of cyclic AMP in the platelet inhibit aggregation by any agent (31,32,33). Cyclic AMP probably prevents mobilization of sequestered internal Ca^{++} which appears to be involved in all platelet functions (33). We do not believe that high endogenous levels of cyclic AMP are responsible for the CH platelet disorder since the ADP response is normal. It will be interesting to learn what the location of the defect is and whether or not it is a block in the arachidonic acid pathway or a more encompassing disorder. One approach to this end is to use high pressure liquid chromatography to study the products sequentially produced along the pathway. One laboratory (34) has successfully employed this to study arachidonic acid metabolism in the platelets of hemophiliacs. In conclusion, the defect in platelet aggregation of CH dogs appears to be in prostaglandin biosynthesis and this must be added to the extensive list of hematologic disorders in canine CH.

Finally, platelet function in heterozygous dogs is only moderately impaired, both in aggregation response to collagen and production of TXB_2. Whether

this is truly a phenotypic expression of CH heterozygosity or an unrelated phenomenon is unknown. However, this observation may have some practical value, since genotype of puppies with heterozygous parents that were not born with grey coats is presently determined by test matings. Testing for platelet aggregation in response to collagen may be a simple and straightforward approach to indicate genotype of littermates.

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VITA

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