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Experimental and Clinical Models of Hematopoietic Stem-Cell Competition

Patrick Sean Sullivan

To the Graduate Council:

I am submitting herewith a dissertation written by Patrick Sean Sullivan entitled "Experimental and Clinical Models of Hematopoietic Stem-Cell Competition." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Comparative and Experimental Medicine.

Ted P. McDonald, Major Professor

We have read this dissertation and recommend its acceptance:

James Brace, Roger Carroll, Donita Frazier, Erby Wilkinson

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

Experimental and Clinical Models of Hematopoietic Stem-Cell Competition

A Dissertation

Presented for the

Doctor of Philosophy Degree

The University of Tennessee, Knoxville

Patrick Sean Sullivan
May, 1994

To the Graduate Council

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Associate Vice Chancellor
and Dean of the Graduate School

DEDICATION

I dedicate this work to my family, who have given me all that anyone can ask of a family an education, a sense of morals, and a sense of pride in oneself My father Darcy, my mother Phyllis, and my brother Michael have respected my individuality, have supported my every endeavor without reservation, have had the good grace to let me make my own mistakes, and have never faltered in their support of me when I made those mistakes

ACKNOWLEDGEMENTS

Above all, I wish to thank Dr Ted P McDonald Dr McDonald sparked my interest in research many years ago, and has been supportive and encouraging ever since His style of graduate education is to be emulated· he gives enough guidance to keep his students on the right path, and gives them enough latitude to make science exciting If I carry with me any of his love of science, careful logic, and scholarly demeanor, I will count my graduate education a success

I also acknowledge Rose Clift and Marilyn Cottrell, who have been invaluable as teachers, coworkers and friends Besides taking care of all of the details that make an experiment come off without a flaw, they deserve special commendation for tolerating my unorthodox housekeeping and for making the laboratory a truly pleasant place to work

I appreciate the time and committement of Drs James Brace, Roger Carroll, Donita Frazier, and Erby Wilkinson Their input into this phase of my education has been thoughtful and timely, and I have known at every step of this process that they held my best interests at heart

I owe a debt to my coworkers on these and other projects Specifically, Drs Mark Freedman, Rebecca Gompf, Carl Jackson, Ted McDonald, and Lynn Schmeitzel have contributed to the manuscripts which comprise this dissertation, and I am indebted for their assitance

Kendra Manning, Wanda Aycock, Ann Vierra, Kim Cline, Deb Haines, and Michelle Vaughn have all made significant contributions to the work presented in this dissertation, and

deserve recognition for their hard work and technical expertise

The administration and faculty of the College of Veterinary Medicine, including Dr Hyram Kitchen (who originally advocated the idea of involving veterinary students in graduate education early in their careers at the College) and Dr Mike Shires, have been consistently supportive of my work, and deserve credit for their efforts to integrate research training and a clinical education.

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ABSTRACT

The inverse relationship between erythropoiesis and thrombocytopoiesis observed in mice held in hypoxia has given rise to an hypothesis of hematopoietic stem-cell competition a strong hormonal call for the production of red blood cells results in increased erythropoiesis at the expense of platelet production In this work, the relevant literature concerning this hypothesis is reviewed, and 5 experimental and clinical studies designed to test the hypothesis are detailed

In experiments with thyroxine, mice made iatrogenically hyperthyroid by administration of exogenous L-thyroxine were found to have increased production of red blood cells, with a concurrent suppression of platelet production Since the stimulatory effect of thyroxine on erythropoiesis is partly mediated by a β_2 adrenergic mechanism, propranolol, an inhibitor, was administered concurrently with thyroxine in other experiments, the results showed a decrease in the level of suppression of thrombocytopoiesis Testosterone, a hormone which stimulates production of red blood cells by promoting proliferation of early hematopoietic stem cells, produced increases in red blood cell and platelet production of castrated mice, with no effect on white blood cell numbers, the results of these experiments suggest that testosterone acts to increase populations of bipotential hematopoietic stem cells, which are capable of producing both red blood cells and platelets Further, administration of thrombopoietin to mice resulted in increased thrombocytopoiesis, with a concomitant decrease in red blood cell production. In these experiments, a positive correlation of thrombopoietin dose and mean megakaryocyte ploidy was documented, and an inverse

relationship between thrombopoietin dose and packed cell volume was reported

In the clinical models of this work, hypothyroid dogs were found to be anemic with increased platelet counts and decreased platelet size, whereas hyperthyroid cats had an increased mean platelet volume when compared to euthyroid cats Additionally, greyhound dogs, which have an erythrocytosis secondary to a defective hemoglobin function, showed a relative thrombocytopenia when compared to non-greyhound control dogs

In summary, the results of these studies indicate that the erythrocytic and megakaryocytic cell lines share a common, bipotential stem cell Selective stimulation of red cell or platelet production occurs at the expense of the sister cell line

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Part 1

Overview, Review of Literature,

Hypothesis

and

Specific Aims

Chapter 1

Overview

Several recent studies show that production of platelets and RBC are inversely related For example, it is well established that hypoxia, a stimulator of erythropoiesis, causes thrombocytopenia in laboratory animals The thrombocytopenia is most likely the result of a reduction in the production of platelets which is caused by a decrease in the number of colony forming units - megakaryocyte (CFU-Mk), early precursor megakaryocytes (small acetylcholinesterase-positive cells, SAChE+), and recognizable megakaryocytes in the bone marrow In all cases, active erythropoiesis was required for the thrombocytopenia, and passive erythrocytosis (i.e hypertransfusion) did not alter platelet production The hypoxia-induced thrombocytopenia was not caused by sequestration of platelets in an enlarged spleen or by expanding blood volumes We speculate that this thrombocytopenia is caused by competition of a precursor cell of the erythrocytic and megakaryocytic cell lines, i.e , marked stimulation of the erythroid cells by erythropoietin causes a decrease in the number of immature megakaryocytes, leading to decreased thrombocytopoiesis. These data are consistent with the findings of Nicola and Johnson, who proposed sequential restriction of the pluripotent stem cell with a terminal bipotential colony forming cell It should also be mentioned that megakaryocytes and erythrocytes have several biochemical similarities, and several clinical conditions point to an inverse relationship between RBC and platelet

production These *in vivo*, biochemical, and clinical data support the hypothesis that megakaryocytes and erythrocytes share a common precursor cell

Chapter 2

Review of Literature

Hypoxia-induced thrombocytopenia was first described in mice by Birks et al [1] who showed that hypoxia caused a severe and persistent thrombocytopenia, resulting in counts that were only one-third of control values after 12 days of hypoxia The thrombocytopenia was not due to splenomegaly or increased blood volume In a follow-up study by Langdon and McDonald [2], it was shown that C3H mice enclosed in silicone rubber membrane chambers with 6-8% O_2 levels for 2 weeks had increased RBC counts (132% of control values) and decreased platelet production, leading to marked thrombocytopenia (16% of control values) Splenectomized mice placed in hypoxia chambers also showed decreased platelet counts and platelet production values when compared to splenectomized control mice that were kept at ambient O_2 tensions Although the evidence was indirect, it was concluded that hypoxia-induced erythrocytosis caused thrombocytopenia via stem-cell competition between the erythrocytic and the megakaryocytic cell lines

Jackson and Edwards [3] evaluated thrombocytopoiesis in rats after exposure to discontinuous hypobaric hypoxia and found a decrease in platelet counts to approximately 50 to 60% of normal by days 12 and 13 Since platelet survival was normal, the finding of thrombocytopenia is consistent with the hypothesis that hypoxia decreases differentiation of precursor cells into the megakaryocytic cell line, resulting in reduced platelet production. Cooper and Cooper [4] confirmed that mice with long periods of hypoxia had decreased

platelet production (40-52% of controls). These workers concluded that severe hypoxia stimulated erythropoiesis, but inhibited thrombocytopoiesis One hypothesis that was offered was that hypoxia directly suppressed the megakaryocyte precursors in the bone marrow In all of these early studies, hypoxia was shown to cause marked thrombocytopenia in laboratory animals [1-4], the thrombocytopenia was associated with reduced platelet production [2,4], while normal platelet survival values were found [3] Therefore, these findings indicate that marked stimulation of RBC by hypoxia resulted in thrombocytopenia

The effect of hypoxia did not appear to be on thrombocytopoiesis directly, since stimulated RBC production was required for reduced platelet production [2,5,6] It was shown that RBC transfusion (passive erythrocytosis) of mice prior to making them thrombocytopenic by injection of a platelet specific antisera did not decrease their rebound thrombocytotic patterns [5] However, when mice were returned to hypoxic environments after being made thrombocytopenic, marked inhibition of platelet production occurred, indicating that it is the hypoxia (and not the presence of elevated RBC) that decreased platelet production in mice [5]. Shaikh and Erslev [6] found that RBC hypertransfusion (which decreases erythropoiesis) resulted in no change in total circulating platelet counts (TCPC) of mice, confirming the conclusion that for decreased thrombocytopoiesis elevated erythropoiesis is required.

Exposure of BALB/c mice (which were reported to have a defective erythropoietin production mechanism [7]) to mild hypoxia resulted in unaltered platelet counts and normal RBC counts [2] However, follow-up studies [8] showed that if BALB/c mice are exposed to severe hypoxia (5.5 to 6 0% O_2) they will produce significant increases in hematocrits,

accompanied by decreases in platelet production However, the responses seen in BALB/c mice were not as great as those observed in C3H mice. As an explanation of this finding [8], it was shown that BALB/c mice have higher P_{50}'s (right shifted O_2 dissociation curves) and lower erythrocyte 2,3-diphosphoglycerate values than C3H mice, indicating a lower hemoglobin O_2 affinity for BALB/c mice Also, greater changes in RBC and platelets were found in male mice than in female mice with the same degree of hypoxia The work explains why BALB/c mice do not become erythrocythemic in mild hypoxic atmospheres, and provides additional evidence that the effects of hypoxia are not directly upon platelets, but require stimulation of erythropoiesis for thrombocytopenia to develop

Additional studies [9] also showed that platelet production in thrombocytopenic mice recovering from hypoxia was reduced when compared to that of mice recovering from thrombocytopenia induced with rabbit anti-mouse platelet serum (RAMPS), probably because of diminished quantities of megakaryocyte precursor cells (small acetylcholinesterase-positive cells, SAChE+) [10] in the marrow of exhypoxic mice. The finding that the number of SAChE+ cells is decreased in mouse marrow by hypoxia [10] provides direct evidence that megakaryocyte precursor cells are fewer

It appears that hypoxia decreases platelet production by action on a precursor cell or a primitive population of megakaryocytes without altering the ability of mice to produce thrombopoietin [11], the factor that stimulates thrombocytopoiesis in immunothrombocythemic mice We found that 24 hrs of hypoxia prior to, or immediately after, stimulation of platelet production by RAMPS reduced platelet production rates 3 days later, this delayed effect indicated that the effect of hypoxia is on a precursor cell or on an

early megakaryocyte However, if thrombocytopoiesis was stimulated by RAMPS injections given at 24 to 48 hrs before exposure to hypoxia, there was little or no effect on platelet production rates These findings demonstrate that hypoxia does not directly influence the differentiated megakaryocyte pool or its production of platelets

The relative number and size of megakaryocytes in both the bone marrow and spleen have been evaluated in mice after hypoxia [12]. The number of megakaryocytes decreased after 14 days of hypoxia by more than 80% in both the bone marrow and spleen Splenic volumes and megakaryocyte concentrations were altered significantly by hypoxia, but the absolute number of splenic megakaryocytes cycled throughout the experiment Petursson and Chervenick [13] reported similar results In their work, the number of nucleated cells in murine bone marrow and spleens was measured by flow cytometry following isobaric hypoxia Our work [12] showed that mean marrow megakaryocyte diameter was increased after 10 days of hypoxia and was inversely related to the absolute megakaryocyte number in the spleen. Changes in megakaryocyte diameter and number with hypoxia suggest a compensatory mechanism for increase in platelet production, which may be regulated separately in the bone marrow and spleen [14]

Other recent studies [15] reveal that the decreased platelet production resulting from prolonged exposure to hypoxia is primarily the result of decreased differentiation of hemopoietic precursors into the megakaryocyte lineage, rather than decreased megakaryocyte DNA content. It was found [15] that higher ploidy classes actually increase as thrombocytopenia becomes more severe in hypoxia. In this study, megakaryocytopoiesis and platelet production were compared in both C3H mice (in which the modal ploidy class is 32N)

and C57/BL mice (in which the modal ploidy class is 16N), by enclosure in hypoxic atmospheres Although hematocrits increased and platelet counts decreased in both strains of mice with time in hypoxic chambers, megakaryocyte and platelet responses of C3H mice differed from those of C57/BL mice in several respects For example, C3H mice had increased levels of 32 and 64N megakaryocytes, whereas C57/BL mice had increased proportions of 16 and 32N cells after exposure to hypoxia

Several studies have shown that hypoxia increases erythropoiesis and decreases thrombocytopoiesis in laboratory animals and that megakaryocytes are reduced in number [3,9,12], as are colony forming units-megakaryocyte (CFU-Mk) [16] in rats exposed for 4 weeks to normobaric hypoxia It was found that hypomegakaryocytic thrombocytopenia was accompanied by decreased CFU-Mk, increased colony forming units-erythroid (CFU-E) and a normal number of burst forming units-erythroid (BFU-E) and colony forming units-granulocyte-macrophage (CFU-GM) [16]

Megakaryocyte size and number were determined [17] utilizing bone marrow from both normal and hypoxic C3H and BALB/c mice The results indicate that normal BALB/c female mice have increased numbers of megakaryocytes, but of smaller size compared to either BALB/c male mice or to both sexes of C3H mice An inverse relationship between the size and number of megakaryocytes was found in both normal and hypoxic mice Therefore, we calculated the total megakaryocyte masses and found that hypoxia causes a significant decrease in total megakaryocyte mass As before, the effect was greater in C3H mice than in BALB/c mice A decrease in the absolute megakaryocyte mass is consistent with the

hypothesis that hypoxia decreases differentiation of hematopoietic precursors into the megakaryocyte series.

The number of SAChE+ cells in the bone marrow of mice was elevated at 2 days of hypoxia [10], but thereafter, their numbers were significantly reduced The reason for the increase in megakaryocyte precursor cells at this early time period is not known, but this finding is most likely related to the biphasic platelet response previously observed [3] in rats We showed [18] that both TCPC and total circulating platelet mass (TCPM) of mice were increased at this early time period, lending support to the claim that it is a true increase in platelet numbers in the circulation However, the percentage of ^{35}S incorporation into platelets and platelet sizes did not show a concurrent increase [18] These results may indicate that the increase in platelet counts at the early time period was not the result of a true thrombocytopoiesis, but may have been due to direct action of an elevated level of erythropoietin (EPO) [19,20] which has been shown to be elevated at the early time period [21] Stress of hypoxia could also increase platelet counts in mice, similar to the mechanism that causes post surgical thrombocytosis

Experiments with hypoxia generate at least three possible explanations of hypoxia-associated thrombocytopenia. The mechanism could be a direct effect of hypoxia on megakaryocytes (less likely given experiments [5] in which hypertransfusion prior to hypoxia exposure prevents the development of thrombocytopenia), a direct effect of erythrocytosis (less likely given the finding [5,6] that passive erythrocytosis induced by hypertransfusion does not decrease platelet production), or some effect secondary to the action of erythropoietin This latter possibility seems most reasonable based on the experimental data

reviewed herein To prove this hypothesis, experiments were undertaken to document the effects of chronic EPO administration on platelet production As mentioned above, previous studies have shown that high doses of recombinant EPO will stimulate platelet production in rodents [19,20], but long-term hypoxia causes thrombocytopenia [1-5,8-11] In an attempt to explain the role of EPO in hypoxia and its role in causing thrombocytopenia, we injected large doses of EPO over a 7-day period and found elevated erythropoiesis with marked thrombocytopenia [22] This finding was recently confirmed in baboons treated with EPO at the rate of 1000 U/Kg twice daily for three days [23] Thrombocytopenia developed 17-19 days later and lasted approximately 2 weeks In our study [22], femoral marrow megakaryocyte size was unchanged, but megakaryocyte number was significantly reduced in mice treated with large chronic doses of EPO Additional studies [22] showed that the decrease in platelet counts observed in EPO-treated mice was not due to increased blood volumes or to splenic sequestration

This progenitor-cell competition model also predicts that increased thrombocytopoiesis should result in anemia Indeed, anemia was found [22] in mice whose thrombocytopoiesis was elevated after an acute thrombocytopenic episode Therefore, elevated thrombocytopoiesis resulted in decreased erythropoiesis Moreover, ^{59}Fe RBC incorporation and RBC mean cell volumes were not elevated in anemic mice, indicating that the decrease in RBC counts and hematocrits was not due to bleeding, but decreased erythropoiesis Therefore, acute thrombocytopenia causes increased thrombocytopoiesis and decreased erythropoiesis

In a study [24] to examine the relationship of anemia and thrombocytosis, it was found that the platelet count increased with erythroid inhibition by actinomycin D in rats made acutely anemic This finding presents another example to illustrate that suppression of erythropoiesis leads to elevated thrombocytopoiesis

The effects described in this review have concentrated on experimental studies, and some attention should be given to clinical implications of this hypothesis Clearly, every patient with stimulated erythropoiesis is not thrombocytopenic, nor are patients under strong thrombocytopoietic stimulus always anemic The results of the studies reviewed herein differ from such clinical scenarios in that. 1) often doses of hormones or duration of exposure to hypoxia were much larger than the physiologic range, 2) treatments were acute in onset, whereas many clinical conditions are often insidious in onset, and thus, may allow for compensation by the marrow, and 3) these studies have utilized measures of thrombocytopoiesis which are much more sensitive than platelet counting, but which are not feasible to evaluate in humans (e g , ^{35}S incorporation into platelets and SAChE+ cell populations) Thus, this hypothesis should not be dismissed based upon the fact that not all clinical conditions show changes in the peripheral blood predicted by the model

In fact, there are several clinical conditions which have blood values that are in agreement with this model. Cyanotic congenital heart disease (CCHD), hypothyroidism in dogs, iron deficiency anemia, and thrombocytosis in post-splenectomy anemia are all examples of diseases which show that RBC and platelet counts are inversely related 1) In CCHD, elevated RBC counts and thrombocytopenia are frequently observed [25] One study [26] revealed that reduction of red cell volume corrected bleeding and platelet function in CCHD

patients, perhaps by increasing platelet production Also, in other patients surgical correction of the cardiac defect in CCHD reduced the hematocrit values and restored platelet counts to normal [26] 2) In human beings with hyperthyroidism, erythrocytosis is commonly found in association with thrombocytopenia [27] The thrombocytopenia of Graves' disease is occasionally severe enough to cause clinically significant hemorrhage 3) Thrombocytosis has been associated with decreased erythropoiesis in iron deficiency anemia [28-32], and of interest is the fact that during the response to iron therapy for anemia, elevated erythropoiesis led to marked thrombocytopenia [33] However, it should be pointed out that prolonged, severe iron deficiency may result in decreased megakaryocyte concentration [34], and in some cases decreased platelet counts [32], presumably a result of exhaustion of the stem cell pool, depletion of iron stores, and/or folate deficiency 4) In a study [35] to evaluate post-splenectomy platelet counts in patients with anemia, persistent thrombocytosis was observed in patients with continuing anemia Therefore, several clinical conditions point to an inverse relationship between RBC and platelet production

Biochemical similarities in erythroid and megakaryocyte precursors also support the stem-cell competition hypothesis For example, Han et al [36] and Bellucci et al [37] found that both erythrocytic and megakaryocytic cell lineages coexpress glycophorin A and glycoprotein IIIa antigens In the most recent study [37], these workers found that the cells that express the antigen were the size of lymphocytes and appeared at an early time of culture. Other workers [38,39] have presented evidence that these lineages also share common transcription factors, indicating additional similarities in cells of the erythrocytic and megakaryocytic cell lines In the work by Romeo et al [38], a specific DNA-binding protein

of the erythrocytic lineage was found in megakaryocytes from human sources Martin et al , [39] using *in situ* hybridization of mouse bone marrow cells and northern blot analysis, found that an erythroid nuclear factor which is important in the regulation of the transcription of globulin in erythrocytes, was also present in megakaryocytes A recent study by Rowley et al. [40] showed that single cells of the human multipotent cell line (K562) express both erythroid and megakaryocyte antigens, i e , glycophorin A and plt-1 antigen These studies taken together indicate that megakaryocyte and erythrocyte precursors share common genetic regulation and structural traits, and probably a common precursor cell

The above findings indicate that hypoxia decreases platelet production by action on an early precursor cell, possibly a bipotential stem cell, and that hypoxia-induced thrombocytopenia is caused by stem-cell competition between the erythrocytic and megakaryocytic cell lines (Figure 1) Nicola and Johnson [41] have studied the production of colony forming cells (CFCs) from pluripotent hematopoietic stem cells, and have reached the same conclusion based upon experimental data of a unique nature These workers harvested pluripotent hematopoietic stem cells, and then monitored the appearance of CFC of various cell lineages *in vitro* They found a consistent pattern of appearance of CFCs in which white blood cell precursors arose sooner in culture than did erythroid or megakaryocytic CFCs They interpreted their data to imply a stereotyped, sequential restriction of the pluripotent state; the terminal CFC in their scheme was a CFC which was bipotential in its ability to produce only red blood cells or macrophages In addition, several other lines of evidence, including data from experiments with EPO, clinical observations and

biochemical data, agree with this conclusion The data presented herein, therefore, support the hypothesis that megakaryocytic and erythrocytic cell lines share a common precursor cell

Chapter 3

Hypothesis

The hypothesis underlying this work is that competition for a finite number of bipotential stem cells results in an inverse relationship between the production of erythrocytes and platelets. Thus, any hematopoietic stimulus that promotes the passage of marrow cells through the megakaryocytic or erythrocytic differentiation series should detract from the production of the non-stimulated cell line Stimuli which result in increased production of colony forming units-spleen (CFU-S), or of the bipotential stem cell, would be expected to result in the increased production of both red blood cells and platelets Further, the hypothesis suggests that production of white blood cells is not affected by the competition effects that are described.

Chapter 4

Specific Aims

In order to test the hypothesis, several specific aims have been identified for this work

1 To evaluate sensitive indices of platelet production in animals with stimulated erythropoiesis in which such erythropoietic stimulation is not attributable exclusively to an erythropoietin-mediated mechanism Two such stimuli are thyroxine and testosterone,

2 To identify appropriate clinical conditions of altered erythropoiesis, and to evaluate thrombocytopoiesis in patients affected with these clinical conditions, and

3 To evaluate erythropoiesis in animals administered thrombopoietin from a human embryonic kidney cell source

In all experiments described in this work, animals were treated according to protocols approved by the University of Tennessee Institutional Committee on Animal Use and Care, and/or the College of Veterinary Medicine Animal Care and Concerns Committee

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Appendix

Part 1

Figure 1

Scheme showing megakaryocytic and erythrocytic cell lines arising from a common precursor cell Major humoral controlling factors for each cell line are also shown.

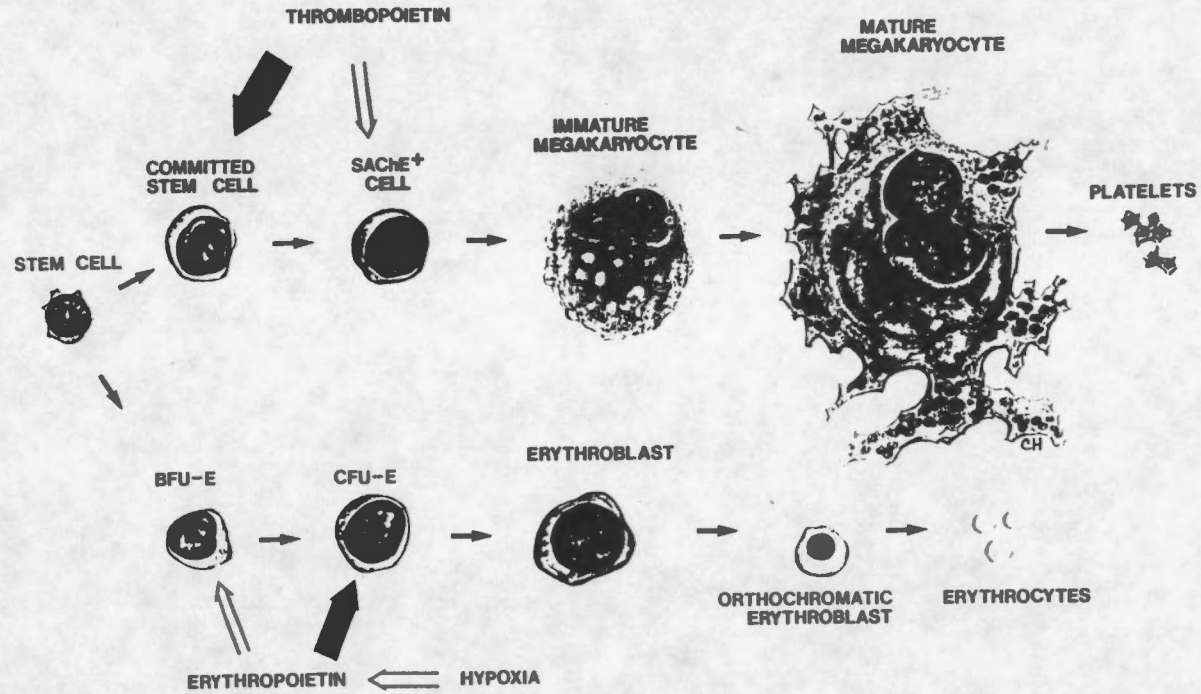
THROMBOPOIETIN
MATURE
MEGAKARYOCYTE
IMMATURE
MEGAKARYOCYTE
COMMITTED
STEM CELL
SACHE+
CELL
PLATELETS
STEM CELL
CH
ERYTHROBLAST
BFU-E
CFU-E
ORTHOCHROMATIC
ERYTHROBLAST
ERYTHROCYTES
ERYTHROPOIETIN
HYPOXIA

Figure 1

Part 2

Effect of Thyroxine on

Erythropoiesis and Thrombocytopoiesis

Chapter 1

Abstract

Thyroxine has been shown *in vitro* to stimulate erythropoiesis by two mechanisms: a direct, β_2 adrenergic receptor-mediated stimulation of red cell precursors, and an indirect, erythropoietin-mediated mechanism Clinical reports have suggested that excess thyroxine also exerts depressive effects on thrombocytopoiesis, but the most sensitive methods of assessing platelet production, i e , $\%^{35}S$ incorporation into platelets, and determination of megakaryocyte size and number, are not appropriate for analysis of platelet production in human patients The purpose of this study was to use a mouse model to investigate the effects of the hyperthyroid state on erythropoiesis and thrombocytopoiesis, and to assess *in vivo* the two mechanisms by which thyroxine has been described to stimulate erythropoiesis *in vitro* We found that thyroxine administration significantly depressed platelet production and stimulated erythropoiesis in mice Both the D- and L-isomers of thyroxine in appropriate doses produced this depression of thrombocytopoiesis, and the effect was dose dependent for both isomers Daily administration of thyroxine increased blood volume, decreased the peripheral platelet count, total circulating platelet count and mass, $\%^{35}S$ incorporation into platelets, and megakaryocyte number and size, and concurrently increased indices of red cell production (packed cell volume, RBC count, total circulating red blood cell count and mass, and reticulocyte count) Additionally, propranolol, a non-specific β-blocker, partially reversed the suppression of platelet production by L-thyroxine, lending credence to the

assertion that the direct, β_2 adrenergic receptor-mediated stimulation of the erythroid cell line by thyroxine reported to exist *in vitro* may also be important *in vivo*

Chapter 2

Introduction

An inverse relationship between erythropoiesis and thrombocytopoiesis has been described in mice responding to an erythropoietic stimulus, i e , hypoxia [1] Long-term hypoxia has been demonstrated to increase erythropoiesis while concurrently decreasing thrombocytopoiesis [2] Platelet counts [1-3], $\%^{35}S$ incorporation into platelets [1-4], colony forming unit-megakaryocyte populations [5], megakaryocyte precursor cells [5-6], and megakaryocyte concentrations in the bone marrow and spleen [7] have all been shown to be suppressed in mice held in an environment with low oxygen tensions, whereas packed cell volumes (PCV) of these animals were increased [1-8] In hypoxic mice, increased erythropoietin (EPO) titers [9] and normal thrombopoietin levels [2] have been demonstrated, supporting the hypothesis [1-2] that the marked stimulation of the erythroid cell line results in competition between precursor cells of the erythrocytic and megakaryocytic cell lines (stem-cell competition) In this hypothesis, increased erythropoiesis leads to decreased numbers of cells in the megakaryocytic cell lineage, leading to decreased thrombocytopoiesis

Thyroid hormones are known to stimulate erythropoiesis *in vitro* [10-12], and clinical states of thyroid dysfunction suggest that thyroxine levels are related to *in vivo* erythropoiesis. In cultures of mammalian marrow, thyroxine increased proliferation of cells in the erythroid series [10,11] The mechanism of this response was proposed to be at least partially mediated by a β_2-adrenergic receptor, as addition of propranolol (a $\beta_1\beta_2$ adrenergic receptor

antagonist) or butoxamine (a selective β_2 adrenergic receptor antagonist) prevented erythroid stimulation *in vitro* [10] Interestingly, this direct stimulatory effect did not seem to be dependent on calorigenic potential, i e , the D-isomer of thyroxine, which has no calorigenic activity, stimulated *in vitro* erythropoiesis as well as the L-isomer [11] There is compelling evidence that thyroxine exerts both a direct (β_2 adrenergic) and indirect (EPO mediated) effect on erythroid precursors [10-14] Because the *in vitro* data have been obtained using cultures of whole marrow, this "direct" effect can only be assigned with certainty to the level of the bone marrow, to assert with certainty that the β-adrenergic receptor is on the erythroid cell precursors would require that similar experiments be done on purified cultures of erythroid cell precursors

Clinical data also support the hypothesis of direct and indirect action of thyroxine by the state of the erythron in thyroid deranged patients in one review, about 20% of cats with hyperfunctional thyroid adenomas had an erythrocytosis [15], and in a study of 56 dogs with hypothyroidism a normocytic, normochromic anemia was observed in about half of the subjects [16] In human beings, hyperthyroidism is associated with increased erythropoiesis and an increased plasma volume, whereas up to 60% of hypothyroid patients are reported to be anemic [17]

The effects of thyroxine on the thrombocytopoietic system are poorly understood The earliest reports of such effects were in the form of case reports of a hemorrhagic tendency in hyperthyroid patients [18-20] Thrombocytopenia in Grave's disease has been proposed to be due to several factors an autoimmune phenomenon, secondary to hypersplenism, or the result of direct damaging effects of thyroid hormones on platelets [21]

Results of a recent study [22], suggested, despite the historical association between autoimmune thrombocytopenia and Grave's disease [23], that the cause of thrombocytopenia in hyperthyroidism is primarily metabolic, although in rare cases an autoimmune thrombocytopenia may be superimposed. Use of platelet volume or size to characterize blood disorders allows detection of more subtle changes in thrombocytopoiesis [24] than platelet count alone. Ford et al [25] have described an increased mean platelet volume in human hyperthyroid patients, and conversely, van Doormal et al [26] have reported that small sized-platelets predominate in the hypothyroid state

These latter reports suggest the need for a study in which very sensitive parameters of thrombocytopoiesis can be utilized to evaluate the hyperthyroid state In mice, platelet counts have been described as a less sensitive index of thrombocytopoiesis than ^{35}S uptake into platelets [27], or megakaryocyte size and number, and percentage of small acetylcholinesterase-positive (SAChE+) cells in bone marrow. It was the goal of this work to determine the relationship between thrombocytopoiesis and the known erythropoietic stimulation induced by the thyroid hormone, and to evaluate the two proposed mechanisms (i.e , direct and indirect) of erythroid stimulation in relation to the effects of thyroxine on the thrombocytopoietic system. The results support the theory that thyroxine concurrently stimulates erythropoiesis and suppresses thrombocytopoiesis *in vivo*, and that the suppression of thrombocytopoiesis by L-thyroxine can be partially blocked by treatment with the non-specific β-adrenergic-blocker, propranolol

Chapter 3

Materials and Methods

Animals Male C3H/HENHSD (C3H) mice purchased from Harlan Sprague-Dawley (Indianapolis, IN) 6-8 weeks of age were used in this work

Pharmaceuticals Thyroxine (L- and D-isomers) from Sigma Chemical (St Louis, MO) in the sodium salt form was dissolved in 1 x 10^{-3} M NaOH (pH 10 3) and administered subcutaneously (s c), 1 x 10^{-3} M NaOH was used as the control substance [28] Propranolol (Inderal, Ayerst Pharmaceutical, New York, NY) was obtained in the intravenous injectable form and diluted with appropriate volumes of 0 9% saline before injecting intraperitoneally (i.p.) Saline (0 9%) was used as the control material for injections of propranolol

Experimental Design In the dose response experiments, the total daily dose of L- or D-thyroxine was divided into two injections and administered s c , mice were treated on days 0 and 1, and were sacrificed on day 3 ` In the time course experiments, mice were given a single daily s c injection of thyroxine at a dose of 25 μg/mouse/day In experiments with propranolol, a daily dose of 4 mg/kg of the β-blocker was divided into two i p injections One injection was administered 1 hr before the daily dose of 25 μg of thyroxine and the other dose of propranolol was given approximately 6 hrs later the same day.

^{35}S Incorporation Single intravenous (i v.) injections of 30 μCi of ^{35}S ($Na_2{}^{35}SO_4$) were given 24 hrs prior to sacrifice, and blood (0 5 - 1 0 ml) was collected from sodium pentobarbital anesthetized mice (0.5 ml of 5 mg/ml sodium pentobarbital administered i.p. five

to ten min before collection) via cardiac puncture Platelets were collected by differential centrifugation at 450 x g and were washed free of red cells and plasma Percentage of ^{35}S incorporation into platelets was then calculated using the method described by McDonald [29]

Platelet Size Samples of blood for platelet sizing analysis were collected by cardiac puncture into 1 0 ml of 3 8% sodium citrate solution following anesthesia with sodium pentobarbital The blood samples were then centrifuged at 22°C for 4 5 min at 160 x g to allow collection of the platelet-rich plasma (PRP) fraction that was used for size analysis using an Electrozone Celloscope (Particle Data, Inc Elmhurst, IL) [26,30] with a 128 multichannel analyzer The instrument was calibrated using 2 02 μm diameter latex particles. When samples were used for both platelet sizing analysis and ^{35}S incorporation, the PRP was added back to the packed RBC and diluted with 1% EDTA-0 538% NaCl solution for a total volume of 3 0 ml, then recentrifuged at 450 x g

Megakaryocyte Size and Number. A femur from each mouse was removed and fixed in 10% phosphate-buffered formalin Bones were embedded in glycol methacrylate and multiple sections, each of 2 μm thickness, were cut at 100 μm intervals from throughout the femur. The sections were stained with hematoxylin and eosin. For megakaryocyte size, a minimum of 200 megakaryocyte profiles were identified and analyzed using a digitizing image analysis system (Analytical Systems, Atlanta, GA) The method of Weibel [31] was used to correct for optically lost caps, and corrections were made for tissue shrinkage as previously described by Cullen and McDonald [32]. For analysis of megakaryocyte number, sections were examined by light microscopy at 400X, and number of megakaryocytes per high

powered field (HPF) was counted in a minimum of ten marrow per femur The average number per HPF was then corrected for optically lost caps and converted to number per unit volume as previously described [32]

Blood Volume Determination and Related Calculations· ^{59}Fe-labelled erythrocytes were used in the determination of the effect of exogenous thyroxine administration on blood volumes [3] Donor mice were given 0 5 μCi each of ^{59}Fe as ferric chloride, 48 hrs later, these mice were sacrificed and the ^{59}Fe-labelled erythrocytes were collected by cardiac puncture into citrate anticoagulant The labelled red cells were isolated from the citrated plasma by centrifugation, and the packed red cells were resuspended in 0 9% saline Control animals treated with daily s c injections of 1 x 10^{-3} NaOH and animals treated with daily s c injections of 25 μg/day of L-thyroxine were injected intravenously with 0 1 ml of ^{59}Fe-labelled red blood cells 15-20 min prior to sacrifice Blood (100 μl) was diluted in 2 0 ml of water, and the radioactivity of the resulting solution was counted in a gamma counter Blood volume is expressed as ml/100 g body weight (%)

Blood volumes were then used in the calculation of absolute measurements of peripheral red blood cells and platelets (i.e , total circulating red cell count [TCRBCC], total circulating red cell mass [TCRBCM], total circulating platelet count [TCPC], and total circulating platelet mass [TCPM])[3] TCRBCC was calculated by multiplying the peripheral red blood cell count by the total ml of blood/mouse, and TCRBCM was calculated by multiplying the TCRBCC by the average erythrocyte size Similarly, TCPC represents the product of peripheral platelet count and total blood volume in ml, and TCPM is calculated by multiplying the TCPC and the average platelet size [3]

Other Hematologic Evaluations A sample of blood collected from the retroorbital sinus was utilized for analysis of platelet count (performed manually using a hemocytometer chamber and phase-contrast microscopy), red and white blood cell counts (performed with a Coulter Counter, Coulter Inc, Hialeah, FL), and reticulocyte count (performed manually on New Methylene Blue-stained smears). Packed cell volumes (PCV) were performed by standard techniques

Statistics The Students t test was used for statistical analysis of data One mouse was considered to be a single data point in determining degrees of freedom

Chapter 4

Results

Dose-Response of Thyroxine $\%^{35}S$ incorporation into platelets of mice was evaluated following administration of doses of 25-200 µg/mouse of both L- and D-thyroxine (Figure 1) The total dose was divided into four subcutaneous injections, given two each on days 0 and 1 of the experiment The vehicle (1×10^{-3} M NaOH) was used as the control substance A dose of 30 µCi of ^{35}S was administered i v on day 2, and mice were sacrificed on day 3 Both thyroxine isomers produced significant depression of thrombocytopoiesis (D-isomer $P < 0\ 005$, L-isomer $P < 0.0005$), as indicated by reduced ^{35}S incorporation into platelets The L-isomer produced greater suppression of ^{35}S uptake than the D-isomer at each dose evaluated, although no statistical significance in these values was found Control data are represented as day 0 in Figure 1

Time Course of Thyroxine Effects Daily administration of 25 µg/mouse/day of L-thyroxine also resulted in significant suppression of platelet production (Figure 2), both platelet count ($P < 0\ 05$ to $P < 0.005$ on days 6 and 8) and $\%^{35}S$ incorporation into platelets ($P < 0\ 05$ to $P < 0\ 005$ on days 2-8) were decreased over the course of 8 days of thyroxine administration (Figure 2A and B) Control animals were dosed daily with a like volume of 1×10^{-3} M NaOH Control animals were sacrificed on each day of the experiment; animals sacrificed on different days did not differ statistically, and are therefore pooled as controls (day 0 in Figure 2) Additionally, platelet size was found to be increased ($P < 0\ 05$) following

8 days of treatment (Figure 2C) and reticulocyte counts (Figure 3A, $P < 0.005$), PCV (Figure 3B, $P < 0.025$), and red blood cell counts (Figure 3C, $P < 0.05$) were also increased

Table I shows that megakaryocyte size, expressed as mean megakaryocyte diameter, was significantly decreased in mice by treatment with L-thyroxine when compared to control levels after 2 - 6 days ($P < 0.05$ to $P < 0.005$) of treatment. Likewise, megakaryocyte concentrations were significantly lower when compared to control levels following thyroxine treatment of 4 - 8 days duration ($P < 0.05$)

Blood Volumes and Related Calculations: Daily administration of 25 µg/mouse/day of L-thyroxine significantly ($P < 0.05$) increased the blood volume of mice following 6 days of L-thyroxine administration (Table II) Daily administration of this dose of thyroxine also significantly depressed TCPC and TCPM ($P < 0.0005$), while TCRBCC and TCRBCM were increased ($P < 0.05$ and $P < 0.005$, respectively) by this treatment (Table III).

Effects of Concurrent Thyroxine and Propranolol Administration In these experiments, 25 µg of L-thyroxine per day were administered s c in a single dose, and two doses of propranolol were administered i p before and after the thyroxine injection (total dose of 16 µg/kg) This injection regimen was followed for four days (days 0-3), control animals received 1×10^{-3} M NaOH and 0 9% saline on an identical injection schedule The animals were then sacrificed, and ^{35}S incorporation into platelets were evaluated. As in previous experiments, both L- and D-isomers of thyroxine significantly (L-isomer $P < 0.0005$, D-isomer $P < 0.05$) suppressed ^{35}S incorporation into platelets (Figure 4), with the L-isomer producing a greater suppression than the D-isomer. Propranolol partially prevented the suppression of platelet production by L-thyroxine ($P < 0.05$) when administered concurrently

with the hormone (L-Thy versus L-Thy/Prop) However, ^{35}S incorporation values did not return to control levels (Figure 4) after concurrent thyroxine and propranolol treatments

Chapter 5

Discussion

This work demonstrates that *in vivo* stimulation of the erythroid cell line by thyroxine is associated with decreased thrombocytopoiesis While indices of erythropoiesis (reticulocyte count, RBC count and PCV) increased following thyroxine administration (Figure 3), thrombocytopoietic activity decreased as measured by platelet count, $\%^{35}S$ incorporation into platelets (Figure 2) and megakaryocyte number and diameter (Table I) The increased platelet size observed following thyroxine administration (Figure 2) is presumed to be a compensatory effort to maintain platelet mass in the face of marked thrombocytopenia This finding is in agreement with reports of increased platelet volume in human hyperthyroid patients [22,25]

Hyperthyroidism has been reported to cause an increase in blood volume in human beings [17], and this report is in agreement with our finding of increased blood volume in mice following administration of exogenous thyroxine. Reticulocyte count, which is expressed as a percentage of total red cells, is independent of blood volume However, this increase in blood volume accounts for the early decrease in the PCV (Figure 3), since an increase in blood volume before a large number of new RBC's enter the peripheral circulation will cause a dilutional effect This confounding factor is eliminated by considering absolute parameters of red cell and platelet production (TCRBCC, TCRBCM, TCPC, and TCPM, Table III). These data show that, independent of changes in blood volume, red cells in the

peripheral circulation are increased in number and mass following several consecutive days of thyroxine administration, while platelet numbers and platelet masses are decreased in the same animals. A consistent feature of these data is that red blood cell appearance in the peripheral blood peaks following 6 days of administration, and then moderates (although remaining significantly elevated as compared to controls) This likely represents a plateau effect of the stimulation of red cell production by thyroxine, the mechanism of which we have not investigated

There are a number of hypotheses which could explain the observed inverse relationship between erythropoiesis and thrombocytopoiesis The two effects could be independent; i e , stimulation of erythropoiesis by thyroxine occurs via the well documented direct and indirect mechanisms already described [10-14], while the hormone simultaneously exerts an independent, direct suppressive effect on some cell population in the marrow, producing the observed peripheral changes in platelets However, given the similar pattern of erythroid and platelet changes already reviewed in naturally occurring thyroid derangement and in hypoxia, it seems likely that the two effects occur by a related mechanism

Two mechanisms have been described for erythroid stimulation by thyroxine [10-12] First, a direct stimulatory effect of thyroxine *in vitro* which has been characterized as a β_2 adrenergic effect [10] This effect appears to be independent of calorigenic potential [11]. The indirect effect of thyroxine is thought to be via the release and action of EPO [14], presumably in response to increased oxygen utilization by cells stimulated by thyroxine. In this work, both L- and D-isomers of thyroxine increased erythropoiesis and suppressed platelet production *in vivo*, although the L-isomer consistently had a greater effect than the

D-isomer This finding is consistent with the hypothesis that *in vivo* the L-isomer exerts both direct and indirect stimulatory effects on the erythroid cell line The latter has been described *in vitro*, whereas the D-isomer, which is incapable of increasing metabolic rate and oxygen consumption of somatic cells, exerts only a direct (β adrenergic) effect Therefore, the D-isomer has a weaker stimulation of RBC production leading to a smaller suppressive effect on thrombocytopoiesis

Concurrent administration of propranolol would be expected to negate the direct effect of thyroxine, and allow only indirect stimulation of the erythron by thyroxine Indeed, administration of propranolol significantly ($P < 0\ 05$) reversed the depression of platelet production produced by L-thyroxine administration (Figure 4, L-Thy vs L-Thy/Prop) Propranolol did not completely prevent the reduced platelet production caused by D-thyroxine, as would be predicted by this model. This effect could be explained by a difference in binding affinities between the L- and D-isomers at the β adrenergic site, different receptor sites for the L- and D-isomers on the red cell precursors, or (less likely) by contamination of the D-isomer with the L-isomer Additional unelucidated mechanisms of erythron stimulation by the D-isomer are possible For clarification, future experiments should utilize a selective β_2-blocker in addition to propranolol The data support the hypothesis that the L-isomer of thyroxine stimulates RBC production by both direct and indirect mechanisms, and that this erythroid stimulation is associated with decreased platelet production

Recent studies [33] have demonstrated a relationship at the molecular level between transcription factors for the erythrocytic and megakaryocytic cell populations. These data further support the idea of a close association between red cell and platelet production in the

marrow Since two experimental states which stimulate erythropoiesis (hypoxia and thyroxine administration) both result in decreased thrombocytopoiesis, and in light of this recent molecular data, a reasonable hypothesis is that there is a common pool of progenitor cells for megakaryocyte and erythroid precursor production. In this hypothesis, an acute and intense demand for production of red cells would result in a depletion of the pool of progenitor cells, leading to decreased thrombocytopoiesis

The definitive answer to this question may lie in the documentation of erythropoietin and thrombopoietin levels in experimentally and/or naturally occurring hypoxic and thyroid deranged patients At this time, sensitive *in vitro* thrombopoietin assays that require microquantities of serum are not available for use in a determination of the hormone To conduct further studies of changes in platelet production in naturally occurring hyperthyroidism, feline patients would be a reasonable study group, since feline hyperthyroidism is most often due to adenomatous hyperplasia of the thyroid tissue [34] This is in contrast to canine and human patients, in whom neoplastic (thyroid adenocarcinoma in canines) or autoimmune (Grave's disease in humans) diseases are often the cause of increased thyroid hormone levels; these diseases can have broader systemic effects and could be confounding factors in such an analysis Further, the reason for the difference in platelet size in hyperthyroid patients should be evaluated more fully to characterize changes in thrombocytopoiesis that occur in this condition [24-26]

This work was intended to explore the relationship between erythroid stimulation by thyroxine (reported to have two distinct mechanisms *in vitro*) and thrombocytopoiesis. We conclude that thyroxine administration, like hypoxia, depresses thrombocytopoiesis while

concurrently stimulating erythropoiesis These data further support the concept [33] that the precursor cells of the erythroid and megakaryocytic lineages bear a close association, the changes described herein are consistent with a common progenitor population that is depleted by the indirect (EPO mediated) and direct (β_2 adrenergic) stimulation of the erythroid cell line in this *in vivo* system

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Appendix

Part 2

Figure 1

The effect of various doses of D- and L-thyroxine on ^{35}S incorporation into platelets of mice. Each data point represents a group of 10 mice, except at 25 μg and 100 μg doses of the D-isomer, where 9 mice were used per point 0 dose value represents data from mice given 1 x 10^{-3} M NaOH (vehicle) Vertical bars indicate S E. Values were significantly different from control $*P < 0\ 05$, $**P < 0\ 005$, $***P < 0\ 0005$.

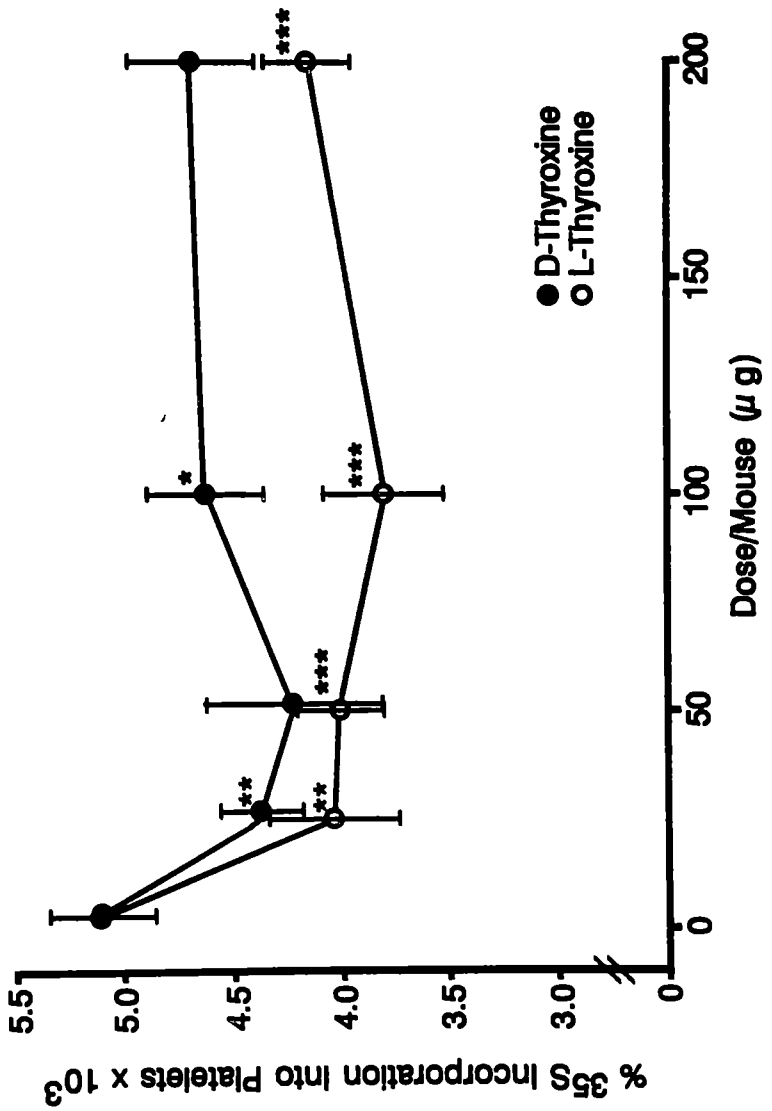
% 35S Incorporation into Platelets x 10^3
5.5
5.0
4.5
4.0
3.5
3.0
0
0
50
100
150
200
Dose/Mouse (μg)
● D-Thyroxine
○ L-Thyroxine
*
**
***

Figure 1

Figure 2

The effects of daily administration of L-thyroxine (25 μg/day) on $\%^{35}S$ incorporation into platelets of mice (Panel A), platelet count (Panel B), and platelet size (Panel C) Day 0 value represents data from mice administered 1 x 10^{-3} M NaOH for 2-8 days All mice, including controls, were sacrificed at the same time In all panels, each data point represents the average of six mice and vertical bars indicate S E Values were significantly different from control $*P < 0\ 05$, $***P < 0\ 005$

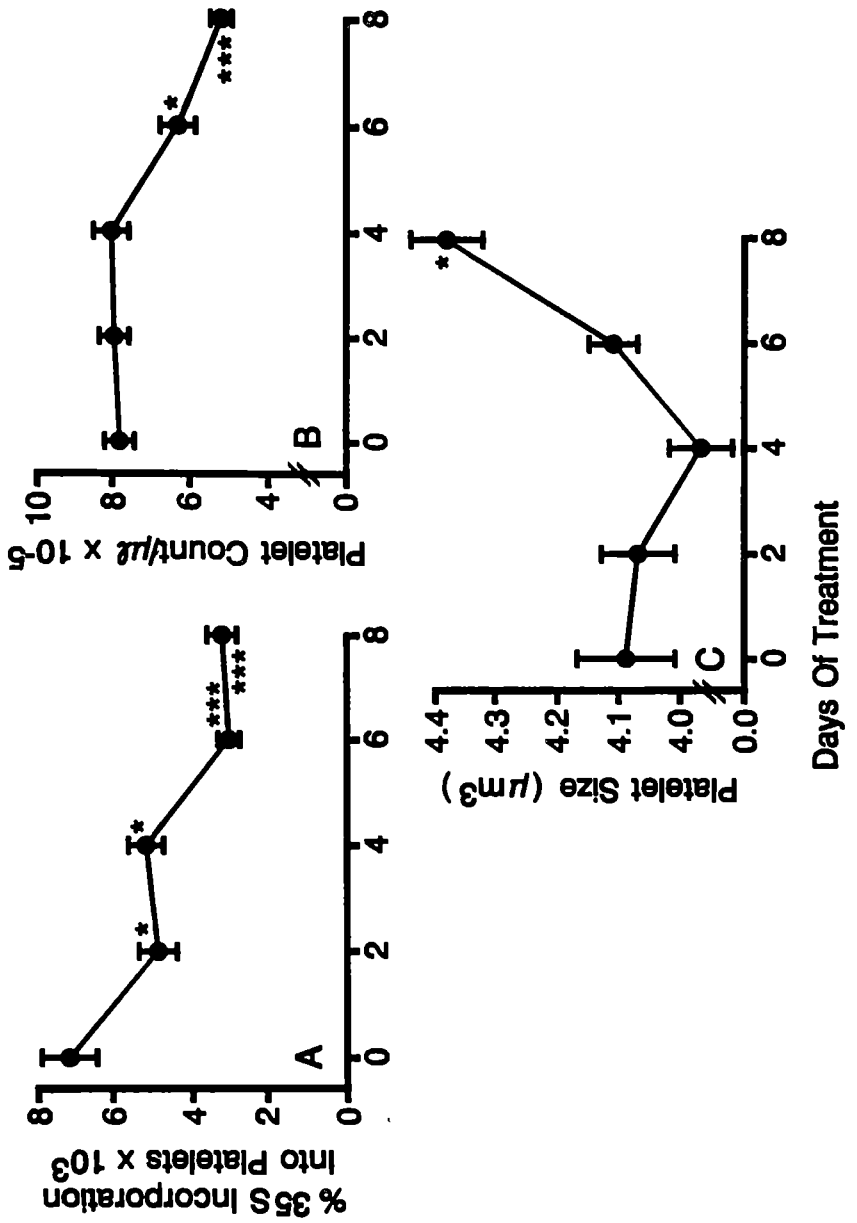
% 35S Incorporation
into Platelets x 10³
Platelet Count/μℓ x 10⁻⁵
Platelet Size (μm³)
Days Of Treatment
A
B
C

Figure 2

Figure 3

The effect of daily thyroxine administration on erythropoietic indices These data are from the same mice as in Figure 2 Panel A shows % reticulocytes, Panel B illustrates packed cell volumes, and Panel C shows RBC counts Day 0 value represents data from mice administered 1 x 10^{-3} M NaOH for 2-8 days. In all panels, each data point represents 6 mice and vertical bars indicate one S E Values were significantly different from control $*P <$ 0 05, $**P < 0$ 025, $***P < 0$ 005

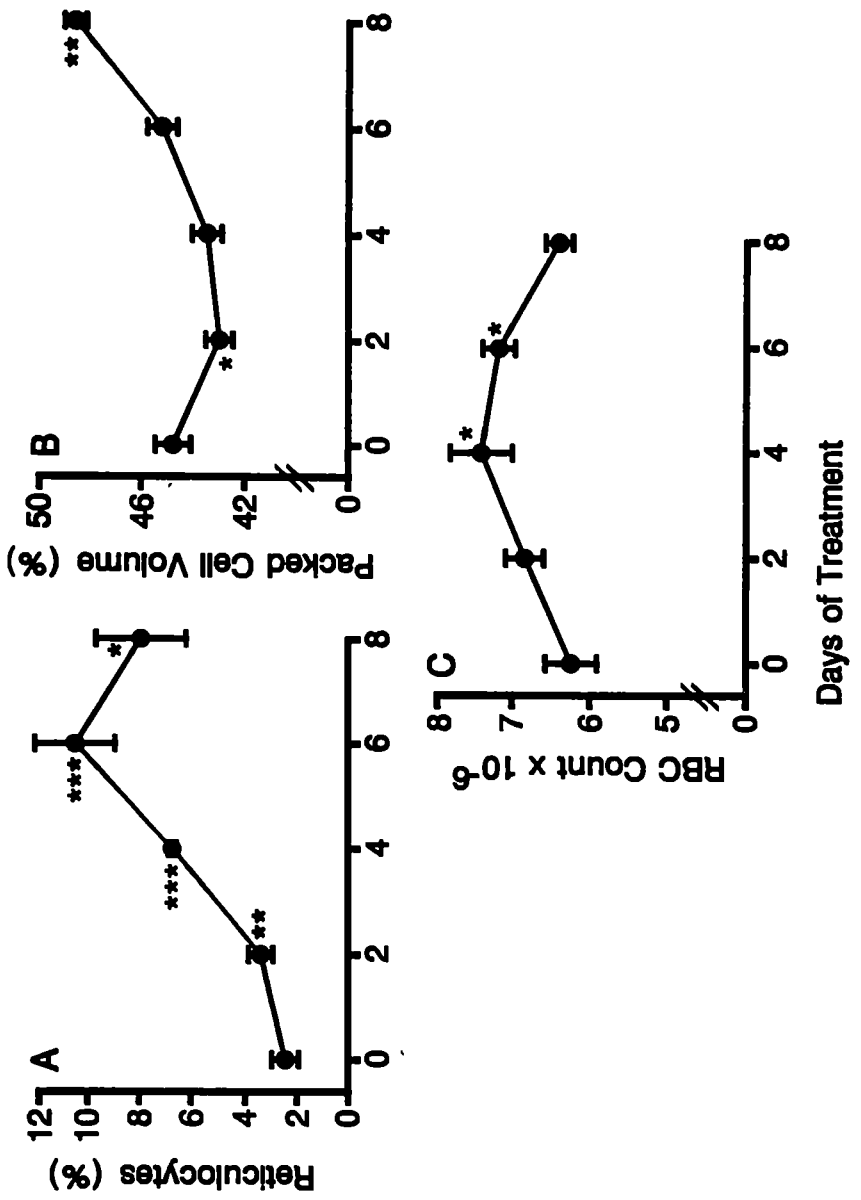
A
Reticulocytes (%)
12
10
8
6
4
2
0
0
2
4
6
8
***
***
**
*
B
Packed Cell Volume (%)
50
46
42
0
0
2
4
6
8
**
*
C
RBC Count x 10^6
8
7
6
5
0
0
2
4
6
8
*
*
Days of Treatment

Figure 3

Figure 4

The effects of 14 days of concurrent thyroxine (25 μg/day) and propranolol (16 μg/day) on ^{35}S incorporation into platelets of mice Twenty-one mice were used for the saline-control group and 5 mice were used for all other treatment groups Vertical bars indicate one standard error Abbreviations used are Prop, Propranolol, L-Thy, L-thyroxine, D-Thy, D-thyroxine Values were significantly different compared to control (saline/10^{-3} M NaOH-treated) mice $*P < 0\ 05$, $**P < 0\ 005$, $***P < 0\ 0005$ Also, L-Thy and L-Thy/Prop were significantly ($P < 0\ 05$) different from one another (not indicated with stars on graph)

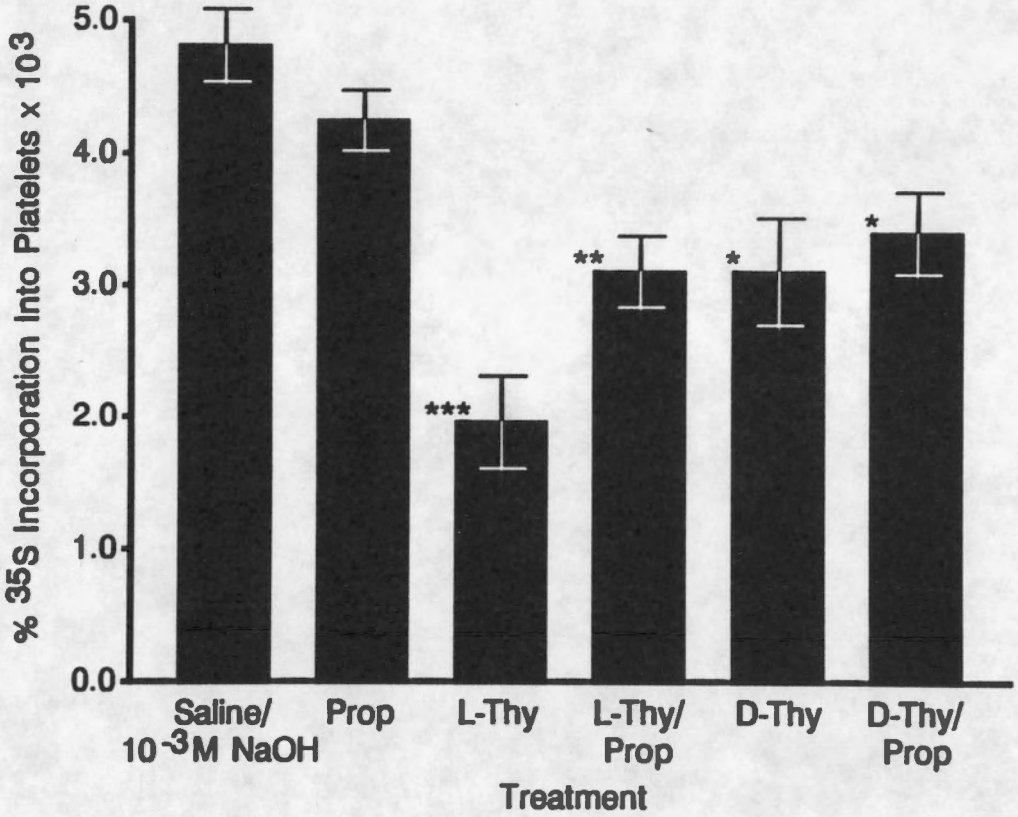
% 35S Incorporation Into Platelets x 10^3
5.0
4.0
3.0
2.0
1.0
0.0
***
**
*
*
Saline/ 10^-3M NaOH
Prop
L-Thy
L-Thy/ Prop
D-Thy
D-Thy/ Prop
Treatment

Figure 4

Table I

Effects of L-thyroxine on megakaryocyte size and number. Values are given as means ± SE (number of mice/treatment) 25 μg of L-thyroxine were injected subcutaneously per mouse per day Megakaryocyte number was corrected for errors due to section thickness, 5% tissue shrinkage, and optically lost profiles [32] Megakaryocyte size, obtained from perimeter measurements of approximately 200 section profiles for each mouse, was corrected for the 5% tissue shrinkage that occurs during histological processing [32] Values were significantly lower than for day 0 * $P < 0\ 05$, ** $P < 0\ 005$

Table I

Days of Treatment	Megakaryocytes/mm^3 x 10^{-3}	Megakaryocyte Diameter (μm)
0	1 83 ± 0 13 (12)	35 69 ± 0 99 (11)
2	1 81 ± 0 05 (7)	32 37 ± 0 99 (9)*
4	1 35 ± 0 05 (7)*	30 39 ± 1 77 (8)*
6	1 39 ± 0 12 (10)*	29 97 ± 1 41 (10)**
8	1 47 ± 0 09 (9)*	33 34 ± 0 91 (10)

Table II

Effects of L-thyroxine on blood volume of mice Values are given as means ± SE 25 μg of L-thyroxine were injected subcutaneously per mouse per day Blood volume was measured by the ^{59}Fe-labeled erythrocyte dilution technique [3] Blood volumes were significantly higher than values found on day 0 $*P < 0\ 05$

Table II

Days of Treatment	Number of Mice	Blood Volume (% of Body Wt)
0	4	5 52 ± 0 16
2	4	5 84 ± 0 40
4	4	5 85 ± 0 27
6	4	6 40 ± 0 33*
8	4	6 13 ± 0 35

Table III

Effect of L-thyroxine on blood platelet and RBC indices Values are given as means ± SE; 4 mice were used in each treatment group 25 μg of L-thyroxine were injected subcutaneously per mouse per day Total circulating platelet counts (TCPC) and total circulating platelet masses (TCPM) were calculated as previously described utilizing platelet counts, blood volumes, and sizes of platelets [3] Total circulating RBC counts (TCRBCC) and total circulating RBC masses (TCRBCM) were calculated as outlined in Methods. Values were significantly different from values for day 0 $*P < 0\ 05$, $**P < 0\ 005$, $***P < 0\ 0005$

Table III

Days of Treatment	Platelet Count x 10^{-6}	TCPC x 10^{-8}	TCPM x 10^{-9} (μm^3)	PCV (%)	RBC Count x 10^{-6}	TCRBCC x 10^{-7}	TCRBCM x 10^{-11} (μm^3)
0	1 05 ± 0 06	13 9 ± 0 6	5 5 ± 0 2	44 8 ± 0 1	7 09 ± 0 11	9 5 ± 0 1	6 00 ± 0 1
2	0 84 ± 0 07	12 0 ± 0 6	4 5 ± 0 2	41 9 ± 0 9*	6 80 ± 0 22	9 8 ± 0 7	6 06 ± 0 1
4	0 73 ± 0 05*	10 7 ± 0 8*	4 1 ± 0 3*	42 6 ± 0 5**	6 68 ± 0 07*	9 7 ± 0 2	6 21 ± 0 1
6	0 62 ± 0 02**	9 6 ± 0 3***	4 1 ± 0 1**	47 3 ± 0 3**	7 00 ± 0 18	11 1 ± 0 4*	7 49 ± 0 1**
8	0 53 ± 0 02***	7 9 ± 0 4***	3 2 ± 0 2***	46 8 ± 0 4**	7 00 ± 0 25	10 5 ± 0 4*	7 10 ± 0 1*

Part 3

Effect of Castration and Subsequent Testosterone Therapy on Thrombocytopoiesis In Male Mice

Chapter 1

Abstract

BALB/c male mice have higher platelet counts than female mice In order to test the hypothesis that testosterone influences platelet production, we evaluated indices of both red blood cell (RBC) and platelet production in intact male BALB/c mice, male mice 4 weeks post-castration, and castrated mice administered maintenance doses of testosterone as testosterone propionate As predicted, castration resulted in decreased hematocrit and body weight in BALB/c mice Body weights and hematocrits returned to noncastrated levels following 2 and 7 days of administration of testosterone, respectively White blood cell (WBC) numbers were unaffected by castration or testosterone administration Additionally, platelet count (956 vs 834 X $10^3/\mu l$), platelet size (3 87 vs 3 75 μm^3), ^{35}S incorporation into platelets (6 36 vs. 4 87 x 10^{-3} %), mean megakaryocyte ploidy (17 43 vs 16 89 N), total circulating platelet mass (TCPM, 490 vs 379 X 10^8 μm^3) and total circulating platelet count (TCPC, 131 vs 103 X 10^7) were significantly ($P < 0\ 05$) decreased in castrated mice compared to intact control mice Administration of daily subcutaneous injections of testosterone (0 5 mg/day) to castrated mice resulted in a return to control (noncastrated) values of mean megakaryocyte ploidy and TCPM (following 2 days of treatment), platelet size, platelet count and TCPC (following 3 days of treatment), and $\%^{35}S$ incorporation into platelets (following 5 days of treatment). Thus, these data support the conclusion that testosterone has a positive influence on thrombocytopoiesis. Whereas late acting stimulators

of erythropoiesis (such as erythropoietin (EPO) and thyroxine) cause competitive reduction in platelet production, in this work stimulation of both red cell and platelet production occurred, with no effect on WBC number The mechanism of the concurrently increased erythropoiesis and thrombocytopoiesis may be due to the stimulation of undifferentiated precursor cells such as a bipotential colony forming cell proposed to contribute to the production of RBCs and platelets

Chapter 2

Introduction

It is well established that gender and sex hormones have an effect on hematopoiesis in mice Male mice of the BALB/c strain are reported to have platelet counts which are higher than their female counterparts [1-3], as well as higher than castrated male mice of the same strain [4] Male BALB/c mice have megakaryocytes which are larger, but less numerous, than the megakaryocytes of female BALB/c mice [2] In C3H mice, another inbred mouse strain, megakaryocyte size and number are the same in males and females, but male mice have a higher mean DNA content (ploidy) in their megakaryocytes than do females [3]. Intact male C3H mice also have higher platelet counts than castrated mice of the same strain [4] Intact female C3H mice were found to have higher $\%^{35}S$ incorporation into platelets than did oophorectomized C3H mice [4] Estrogen treatment has also been shown to increase megakaryocytopoiesis in mice [5] One hypothesis which explains such differences in thrombocytopoiesis in male and female mice is that testosterone positively regulates the production of platelets

Recent work has documented that in mice with 7 different genetic constitutions (crosses and backcrosses of C3H and C57/BL parents), male mice had higher platelet count [6] and megakaryocyte ploidy [7] than female mice of the same genetic makeup The authors concluded that the maternal contribution to the genomic complement of offspring had a more pronounced effect on platelet production than did the contribution of the male parent [6,7],

this phenomenon is known as genomic imprinting, and implies that factors other than simple testosterone levels bear on the relationship between gender and the level of thrombocytopoiesis

The effects of testosterone on red blood cell (RBC) production are more completely characterized Testosterone stimulates the production of RBCs by two apparent mechanisms a direct effect on the production of the pluripotent stem cell (CFU-S), which can be demonstrated *in vitro* [8] and *in vivo* [9], and an indirect stimulation which occurs via erythropoietin production [10,11] For these reasons, androgen therapy has been advocated for the treatment of patients with anemia of chronic renal failure [12]

Androgen effects on erythropoiesis also appear to have physiologic significance in the maintenance of RBC production in healthy individuals, for example, castration of human males results in a decrease in RBC mass of approximately 6% [13], and women given androgens for the therapy of breast carcinoma experience increases in hematocrit of approximately the same magnitude [14] The effect of testosterone in the maintenance of RBC production may be different in males and females, when testosterone was administered to mice in hypoxia, it had a significant stimulatory effect on erythropoiesis in female mice, with no effect in male mice [15]

The goal of this work was to evaluate the "maintenance" role of testosterone in the production of platelets, and to evaluate the interaction of the effects of testosterone on the erythropoietic and thrombopoietic systems To this end, we evaluated sensitive indices of platelet production and red cell indices in intact male mice, castrated male mice, and castrated mice administered replacement testosterone therapy The results indicate that testosterone

has a significant role in the regulation of platelet production in mice

Chapter 3

Materials and Methods

Animals: BALB/c male mice aged 4-5 weeks were obtained from Harlan Sprague Dawley (Indianapolis, IN) Castrated mice were purchased castrated from the company, and in all cases were castrated at least 4 weeks prior to assay Animals were housed in the Laboratory Animal Facility at the University of Tennessee, with a maximum of 10 mice per cage for the duration of the experiment

Testosterone: Testosterone in the form of testosterone propionate (Sertis Laboratories, Phoenix, AZ) was utilized in this work Because the testosterone was supplied in a sesame seed oil carrier, sesame seed oil (Sigma, Indianapolis, IN) was used as a control substance

Experimental Design: Castrated male mice were injected with 0 5 mg/day/mouse by subcutaneous route for 2, 3, 5, or 7 days prior to sacrifice The total daily dose was diluted with sesame oil to a final concentration of 5.0 mg/ml (0 1 ml/injection) Castrated control animals received injections of sesame seed oil only on an identical schedule In all mice in which ^{35}S incorporation into platelets was determined, an intraperitoneal injection of 30μCi of ^{35}S, as $Na_2{}^{35}SO_4$, was given 24 hrs prior to sacrifice

Blood Volume Determination and Related Calculations: For blood volume experiments, 6 normal male BALB/c mice were used as erythrocyte donors [16] These mice were administered 0 5μCi each of ^{59}Fe as ferric chloride by intraperitoneal injection, and 48

hrs later were sacrificed ^{59}Fe labeled erythrocytes were collected into 0 9% sodium citrate anticoagulant, and were isolated by centrifugation. The labeled RBCs were then resuspended into 0 9% saline Control animals treated with sesame seed oil and experimental animals treated with testosterone as described above were then injected intravenously with 0 1 ml of ^{59}Fe-labeled erythrocytes These mice were then sacrificed 15-20 minutes later At the time of sacrifice, 100 µl of blood was collected by retroorbital venipuncture, and this aliquot of blood was diluted into 2 0 ml of water The radioactivity of the resulting solution was then measured by a gamma scintillation counter. The blood volume is expressed as ml/100 g of body weight (%) Blood volumes were then used in the calculation of absolute RBC and platelet indices Absolute indices were calculated as follows [17]

Total Circulating Platelet Count (TCPC) = [Platelet count (/ml)] X [Blood volume (ml)]

Total Circulating Platelet Mass (TCPM) = TCPC X [Mean Platelet Volume(μm^3)]

Total Circulating RBC Count (TCRBCC) = [RBC count(/ml)] X [Blood Volume (ml)]

Total Circulating RBC Mass (TCRBCM) = TCRBCC X [Mean RBC Volume (μm^3)]

Sacrifice of Animals: At the time of sacrifice, 2 5 mg/mouse of sodium pentobarbital (Butler, Columbus OH) in a solution containing 50 units of heparin (Elkins-Sinn Inc, Cherry Hill, NJ) was administered as 0 5 ml of a 5 mg/ml solution by the intraperitoneal route After the mice were in a deeply anesthetized plane (5-10 minutes after pentobarbital injection), a small aliquot of blood was collected by retroorbital venipuncture, this sample was utilized for determination of platelet count, RBC and WBC count, reticulocyte count, and packed cell volume (PCV) An additional 0 5-1 0 ml of blood was collected by cardiac puncture, this latter sample was drawn directly into 1 0 ml of sodium citrate anticoagulant This sample of blood was used for determination of platelet size and of ^{35}S incorporation into platelets.

Immediately post-mortem, one femur and two tibias were collected from each mouse and processed as described subsequently

Hematology: RBC counts and white blood cell counts were performed using a Coulter Counter (Coulter, Hialeah, FL) Platelet counts were performed manually using phase microscopy PCV was determined using a standard microtechnique Reticulocyte counts were obtained by counting the total number of reticulocytes among 500 RBCs, following staining with New Methylene Blue

Platelet Size: Samples for analysis of platelet size [18] were taken from the platelet rich plasma (PRP) obtained as described above For each mouse, a 5 µl aliquot of PRP was diluted into 10 0 ml of isotonic buffered saline, and a sample of the platelets was analyzed with an Electrozone Celloscope (Elmhurst, IL) equipped with a 128 multichannel analysis unit A 48 µm orifice was used, and the instrument setting were Log 10, Gain 4 5, and Current 1 Frequent calibrations were performed using 2 02 µm diameter latex beads. Data are reported as mean platelet volume in μm^3 following normalization of the data

Determination of ^{35}S Incorporation Into Platelets: Platelets from the cardiac venipuncture blood sample were obtained by differential centrifugation of the blood at 450 x g for 4 5 minutes at 22°C Platelets from each mouse were then washed with 0 5 ml of 1 0% ammonium oxalate, then with 1 0 ml of 0 9% saline, and were subsequently resuspended in 0 3 ml of saline The platelet suspension was then counted by diluting 5µl of the suspension in 10 0 ml of isotonic buffered saline and counting total platelets using an Electrozone Celloscope (Particle Data, Inc, Elmhurst, IL) Two 100 µl aliquots of this platelet suspension were then diluted into 5 0 ml aliquots of scintillation fluid [16] The radioactivity

of these latter dilutions was then measured using a beta scintillation counter Percent ^{35}S incorporation into platelets was then calculated as previously described [19].

Megakaryocyte Ploidy Analysis: For measurement of megakaryocyte DNA content [20,21], a sample of marrow was collected by flushing a femur and two tibias with 1.0 ml of a solution of CATCH media The CATCH media contained citrate, adenosine and theophylline in Hanks medium The media was free of calcium and magnesium, and was supplemented with bovine serum albumin and DNAse The marrow suspensions were collected into plastic tubes to prevent megakaryocyte adhesion to glass surfaces The marrow suspensions were filtered through a membrane with 105 μm pores (Small Parts Inc, Miami, FL) The cells were then incubated with a saturating concentration (total 5 μl/ sample) of a rabbit anti-mouse platelet serum (RAMPS) [22] for 30 min at 4°C Each sample was then washed three times with 3 0 ml/wash of CATCH medium Cell suspensions were pelleted between washes by centrifugation for 5 mins at 160 x g in a 4°C centrifuge Each sample was then incubated with 10 μl of fluoresceinated goat anti-rabbit immunoglobulin $F(ab')_2$ fragments (TAGO, Burlingame, CA) for 30 min at 4°C, and washed 3 additional times with CATCH medium at 22°C The FITC labeled cells were then resuspended in 3 0 ml of a solution of propidium iodide (50 μg/ml) The marrow cells were analyzed on an EPICS 753-Flow Cytofluorometer (Coulter Electronics, Hialeah, FL) From 300-800 x 10^3 propidium iodide positive cells were examined per sample, of the propidium iodide positive cells analyzed, the DNA content of from 643 to 2213 (average 1263) RAMPS-positive cells was analyzed Total numbers of cells corresponding to each ploidy class were calculated from the cytofluorometer, and data are reported as the geometric mean of the data for each mouse.

Statistics: Analysis of variance was completed for each response variable using PROC GLM of SAS (SAS Institute, Cary, NC) When the treatment levels were considered as class variables, a total of 7 treatment levels were compared using Dunnett's t-tests, with an experimentwise P value set at 0 05 In addition, linear regression analyses were performed, also using the PROC GLM software, but declaring all treatment levels as numeric (interval) In this analysis, normal mice could not be included, as there was no single numeric representation of their treatment (i e , number of days) A P value of less than 0 05 was considered to be significant

Chapter 4

Results

The results of castration and testosterone supplementation on RBC production were in agreement with previous studies Castration resulted in significant ($P < 0\ 05$) decreases in body weight, PCV and in TCRBCM and TCRBCC (Figures 1-4), however, RBC counts, MCV, and reticulocyte count were not changed by castration (Figure 5, RBC count and MCV data not shown) When compared to castrated mice, mice that had been castrated and supplemented with testosterone at a dose of 0 5 mg/day showed decreases in PCV following 2,3, and 5 days of testosterone replacement, but an increase in PCV was found after 7 days of supplementation (Figure 2) Because testosterone is known to cause increases in blood volume, we also calculated the absolute indices of RBCs (TCRBCM and TCRBCC). As compared to castrated mice, mice castrated and then treated with testosterone showed increased TCRBCM at 3,5 and 7 days following initiation of therapy (Figure 3) TCRBCC count was elevated following 5 days of testosterone therapy (Figure 4) Reticulocyte counts were increased in castrated mice administered testosterone for 3 or 5 days, as compared to both intact and castrated control animals (Figure 5)

Additionally, significant linear relationships existed between days of testosterone administration and PCV, reticulocyte counts, TCRBCC, and TCRBCM The F values, P values, and estimates for intercept and slope of these relationships are depicted in Table I

Castration alone had significant effects on all indices of platelet production evaluated

Castration decreased platelet count (Figure 6), mean platelet volume (Figure 7), $\%^{35}S$ incorporation into platelets (Figure 8), TCPC (Figure 9), and TCPM (Figure 10) Mean megakaryocyte ploidy was also decreased by castration (Figure 11)

Supplementation of castrated mice with testosterone at a daily dose of 0 5 mg/day resulted in increases in platelet indices Platelet counts were returned to normal (precastration) levels by 5 or 7 days of testosterone replacement therapy Similarly, platelet size was returned to control (uncastrated) levels by 3 days of testosterone therapy, and was increased above levels of uncastrated mice by 5 days of treatment with testosterone (Figure 7) Percent ^{35}S incorporation into platelets was returned to normal levels in castrated mice following 5 days of testosterone therapy (Figure 8) TCPC was corrected to levels of uncastrated mice by 3, 5 or 7 days of testosterone injections (Figure 9) TCPM was returned to control levels after 2 days of testosterone therapy, and rose above levels of uncastrated controls on day 5 (Figure 10) Mean megakaryocyte ploidy was increased above levels of uncastrated control mice at the earliest time studies (following 2 days of treatment, Figure 11)

Significant liner correlations were demonstrated between days of testosterone therapy and platelet count, platelet size, TCPC, TCPM and $\%^{35}S$ incorporation into platelets (Table II) The F-values, *P* values, intercept and slopes for these relationships are illustrated in Table II

WBC numbers were not affected by castration or by testosterone therapy (Figure 12)

Chapter 5

Discussion

These data provide evidence that testosterone has a significant effect on the maintenance of platelet counts in male mice This observation is of interest, as there is not general agreement regarding the effect of sex on resting platelet counts in human beings While there is anecdotal evidence that men have higher platelet counts than women, there has been no study which has substantiated this observation in a rigorous fashion Observations of sex-related differences in the platelet counts of mice [1,4,6] are possible since inbred strains of mice have smaller variation in platelet counts attributable to genetics, environment and behavior, therefore, the effect of sex hormones will contribute more heavily to the total variation in platelet counts observed in mice than in human beings

The mechanism of action of androgens on the production of platelets is not known Two recent studies have demonstrated that genomic imprinting plays a role in determining megakaryocyte ploidy [7] and platelet counts [6] of offspring in cross-strain breedings, with the maternal contribution being dominant The effects of testosterone may be involved in the expression of genetic differences, but there are likely independent contributions of these genomic elements and endogenous testosterone levels

Given the documented effect of androgens on population of hematopoietic stem cells [8,9], it seems logical that one mechanism of action of testosterone would be to increase stem cell populations and thereby contribute to the production of both RBCs and megakaryocytes

This effect would also predict decreases in WBC counts following castration, and higher WBC counts in male mice as compared to female mice, neither of these observations have been reported, either in previous studies or in the current work This may be attributable to the fact that the hematopoietic stem cells giving rise to the production of blood platelet and RBCs appear to bear a common lineage [23], therefore, testosterone may by some unelucidated mechanism selectively stimulate the production of the proposed bipotential stem cell [24] in preference to the production of immature precursors of the WBC lineage It seems likely that our ultimate understanding of the scheme of hematopoiesis will include developmental designations not yet characterized by morphological or immunochemical means

Another possibility, which may be operative in conjunction with increases in the bipotential stem cell, would be a testosterone-stimulated production of thrombopoietin (in a manner analogous to stimulation of erythropoietin production) This possibility cannot be evaluated by the data in the current study Since the current methodology for assaying thrombopoietin activity is by mouse bioassay [19], this question could be answered by injecting the plasma of castrated mice and castrated mice administered testosterone into immunothrombocythemic mice and evaluating $\%^{35}S$ incorporation Injected testosterone should not interfere with such an analysis (provided that test plasma was obtained several hrs after testosterone treatment) since testosterone has a very short half life in the plasma.

The observation of simultaneous stimulation of erythropoiesis and thrombocytopoiesis is also of interest given recent observations of an inverse relationship between the production of platelets and RBCs attributed to a competition for hematopoietic stem cells [23] The

current observations are reconciled with this hypothesis in light of the fact that the erythropoietic stimuli reported to stimulate erythropoiesis at the expense of thrombocytopoiesis are stimuli which are relatively late acting factors (relative to pluripotent stem cell populations) such as hypoxia-induced erythropoietin production and thyroxine therapy. This observation also lends credence to the hypothesis that testosterone acts to increase stem cell populations *in vivo*, as a sole erythropoietin-mediated mechanism should result in a thrombocytopenia similar to that observed in mice held in hypoxia [25,26] or administered thyroxine [17]

Nicola and Johnson [24] have proposed that the pluripotent hematopoietic stem cell undergoes a series of restrictions, such that the most immature colony forming cells (CFC) still possess essential pluripotentiality, whereas precursor cells in temporally later maturational stages have restricted potentiality (Figure 13) According to their data, which is based upon temporal patterns of the formation of CFCs of various lineages, megakaryocytes and erythroid precursors are the latest maturationally, and share a terminal bipotential stem cell [24] The data in the present work suggest that testosterone stimulates increases in numbers of the bipotential stem cell (indicated in Figure 13), since no changes in WBC counts were found in castrated mice administered testosterone (Figure 12)

This latter hypothesis allows a retrospective reevaluation of the reports of Byron [8,9], in which testosterone was found to increase CFU-S *in vitro* and *in vivo*. Since the methodology used by Byron to evaluate CFU-S formation was based upon counting erythroid colonies in the spleens of mice following sublethal irradiation and subsequent marrow infusion, the endpoint measured in this previous work is actually the total number of CFU-S

plus the total number of CFC of any restriction state, including the bipotential CFC for erythroid and megakaryocytic precursors Thus, the proposal that testosterone's early effects on hematopoietic stem cells may actually be on the bipotential stem cell is consistent with Byron's earlier reports [8,9] of increased CFU-S following testosterone administration

In summary, castration of male mice results in decreased platelet production, and supplementation of castrated mice with testosterone results in a return to normal levels of thrombocytopoiesis Whereas late acting upregulators of erythropoiesis (such as erythropoietin, thrombopoietin, and thyroxine) cause a competition effect between erythroid and megakaryocytic precursors, testosterone caused a stimulation of both RBC and platelet production in castrated mice Further studies to elucidate the mechanism of these effects are needed, but it seems likely that testosterone stimulates production of the bipotential CFC previously proposed by Nicola and Johnson [24]

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Appendix

Part 3

Figure 1

The effects of castration and testosterone therapy on the body weight of male BALB/c mice Means are indicated by wide vertical bars, with one S E indicated by the smaller bar Means represent a total of 25, 36, 18, 10, 14, and 20 mice for treatments uncastrated, 0 (castrated), and 2,3,5 and 7 days of testosterone therapy, respectively Significance ($P < 0\ 05$) is indicated by the following symbols *, significantly different from mean for uncastrated mice by Dunnett's t-tests, †, significantly different from mean for castrated mice receiving no testosterone therapy by Dunnett's t-tests

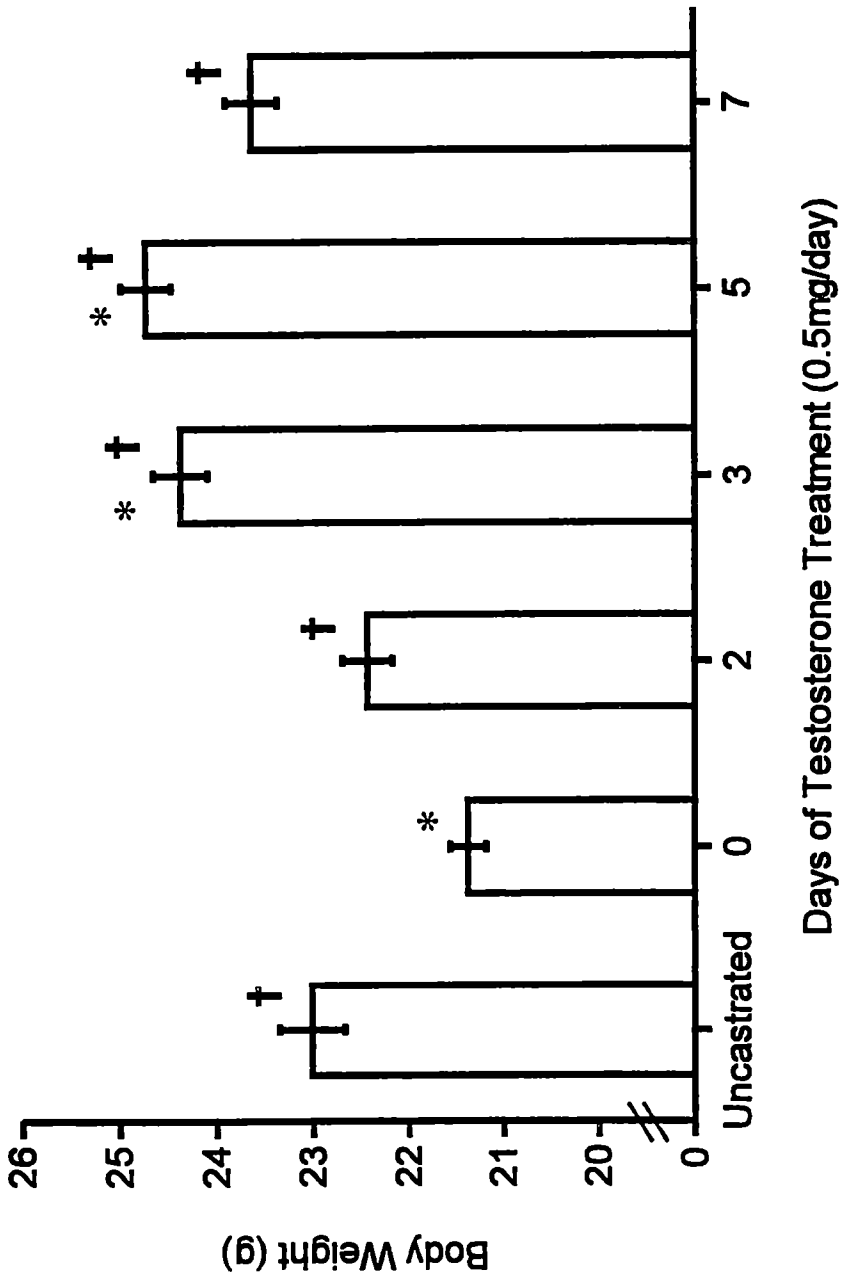
Body Weight (g)
26
25
24
23
22
21
20
0
Uncastrated
0
2
3
5
7
Days of Testosterone Treatment (0.5mg/day)
*
*
*
†
†
†
†
†

Figure 1

Figure 2

The effects of castration and testosterone therapy on the packed cell volume (PCV) of male BALB/c mice Means are indicated by wide vertical bars, with one S E indicated by the smaller bar Means represent a total of 25, 36, 18, 10, 14, and 20 mice for treatments uncastrated, 0 (castrated), and 2,3,5 and 7 days of testosterone therapy, respectively Significance ($P < 0\ 05$) is indicated by the following symbols *, significantly different from mean for uncastrated mice by Dunnett's t-tests, †, significantly different from mean for castrated mice receiving no testosterone therapy by Dunnett's t-tests

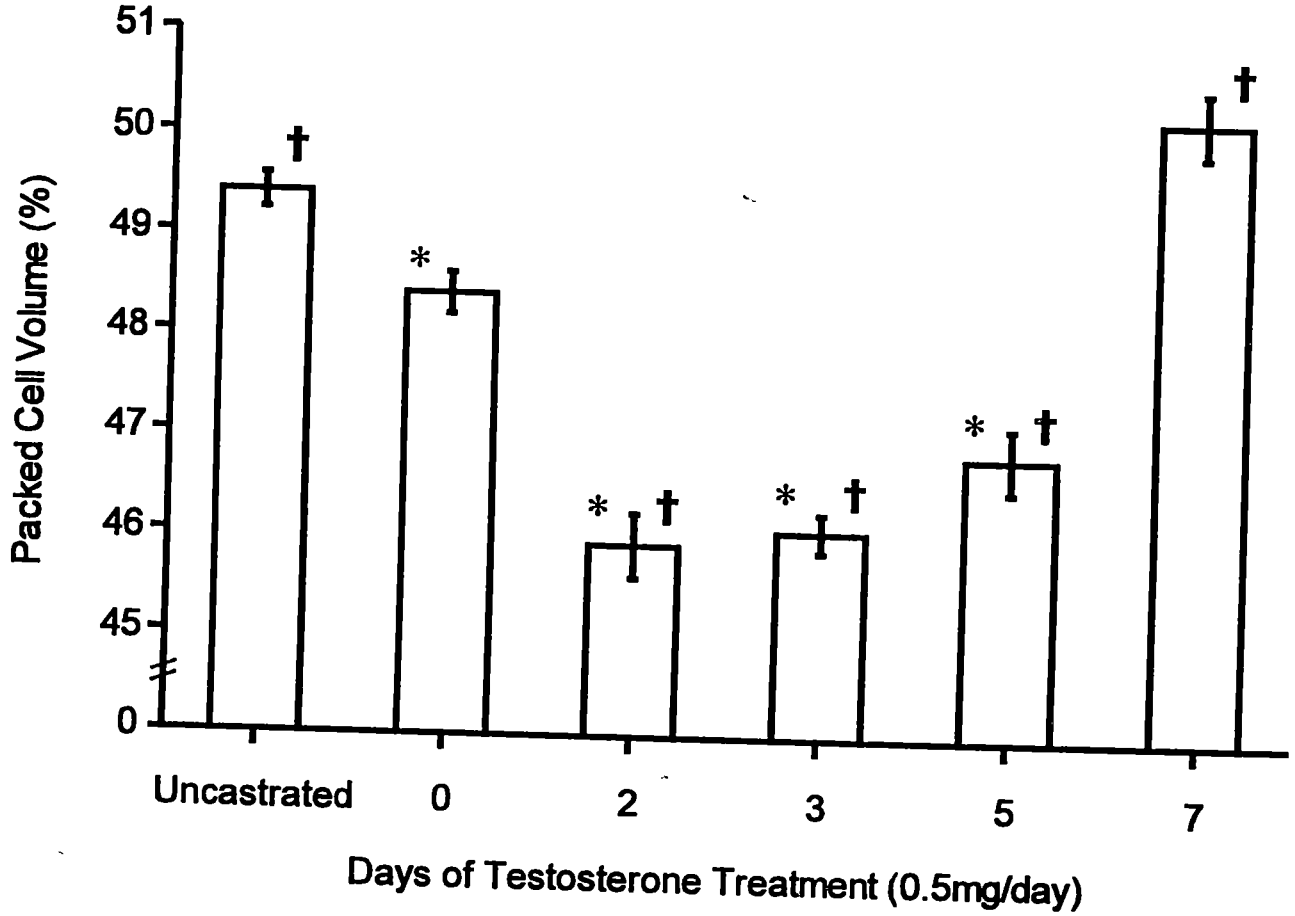
Packed Cell Volume (%)
51
50
49
48
47
46
45
0
Uncastrated
0
2
3
5
7
Days of Testosterone Treatment (0.5mg/day)

Figure 2

Figure 3

The effects of castration and testosterone therapy on the total circulating red blood cell mass (TCRBCM) of male BALB/c mice Means are indicated by wide vertical bars, with one S.E indicated by the smaller bar Means represent a total of 20, 25, 5, 10, 14, and 10 mice for treatments uncastrated, 0 (castrated), and 2,3,5 and 7 days of testosterone therapy, respectively Significance ($P < 0\ 05$) is indicated by the following symbols *, significantly different from mean for uncastrated mice by Dunnett's t-tests, †, significantly different from mean for castrated mice receiving no testosterone therapy by Dunnett's t-tests

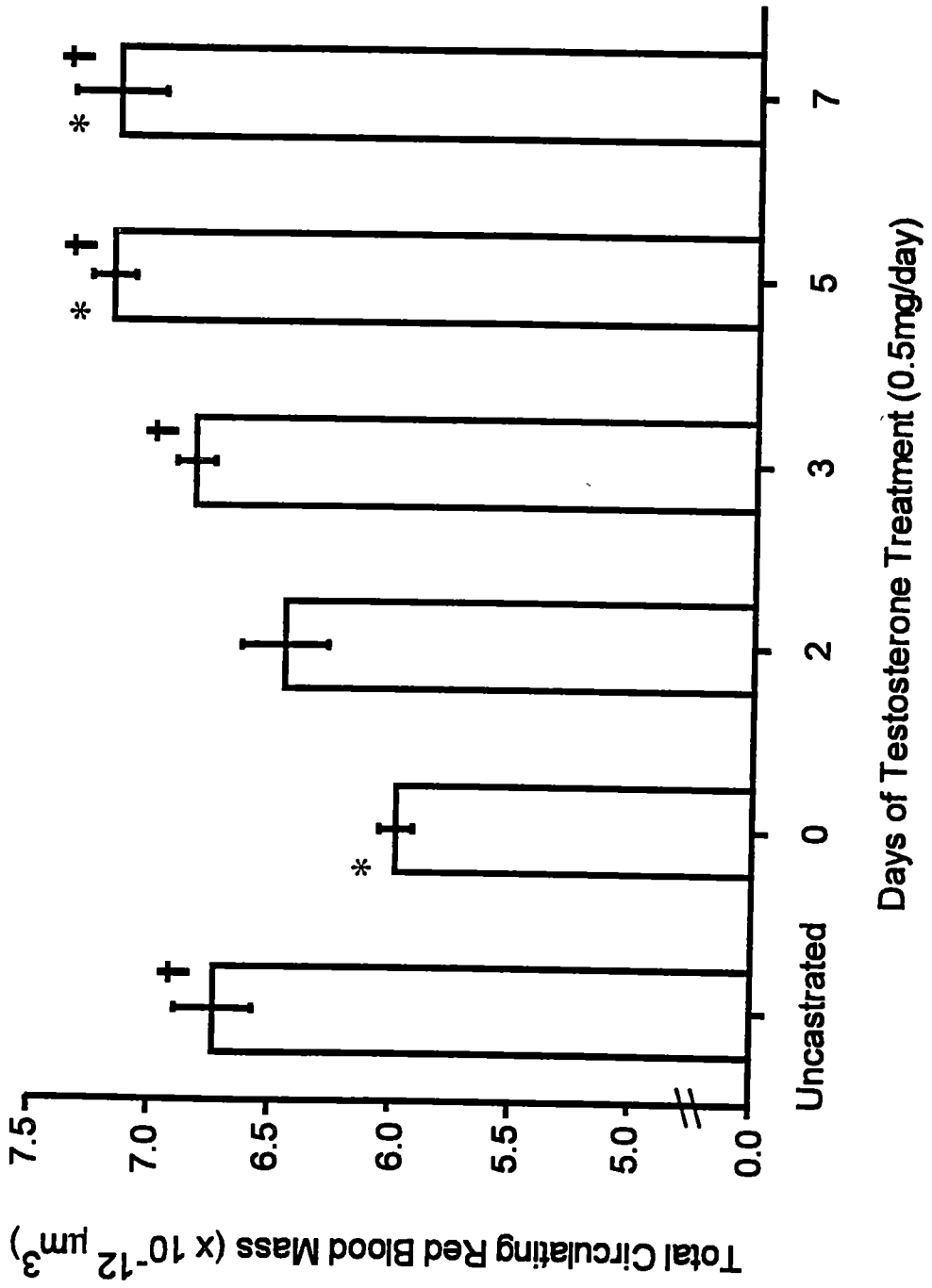
Total Circulating Red Blood Mass (x 10-12 µm3)
7.5
7.0
6.5
6.0
5.5
5.0
0.0
Uncastrated
0
2
3
5
7
Days of Testosterone Treatment (0.5mg/day)

Figure 3

Figure 4

The effects of castration and testosterone therapy on the total circulating red blood cell count (TCRBCC) of male BALB/c mice Means are indicated by wide vertical bars, with one S E indicated by the smaller bar Means represent a total of 20, 25, 5, , 14, and 10 mice for treatments uncastrated, 0 (castrated), and 2,3,5 and 7 days of testosterone therapy, respectively Significance ($P < 0\ 05$) is indicated by the following symbols *, significantly different from mean for uncastrated mice by Dunnett's t-tests, †, significantly different from mean for castrated mice receiving no testosterone therapy by Dunnett's t-tests

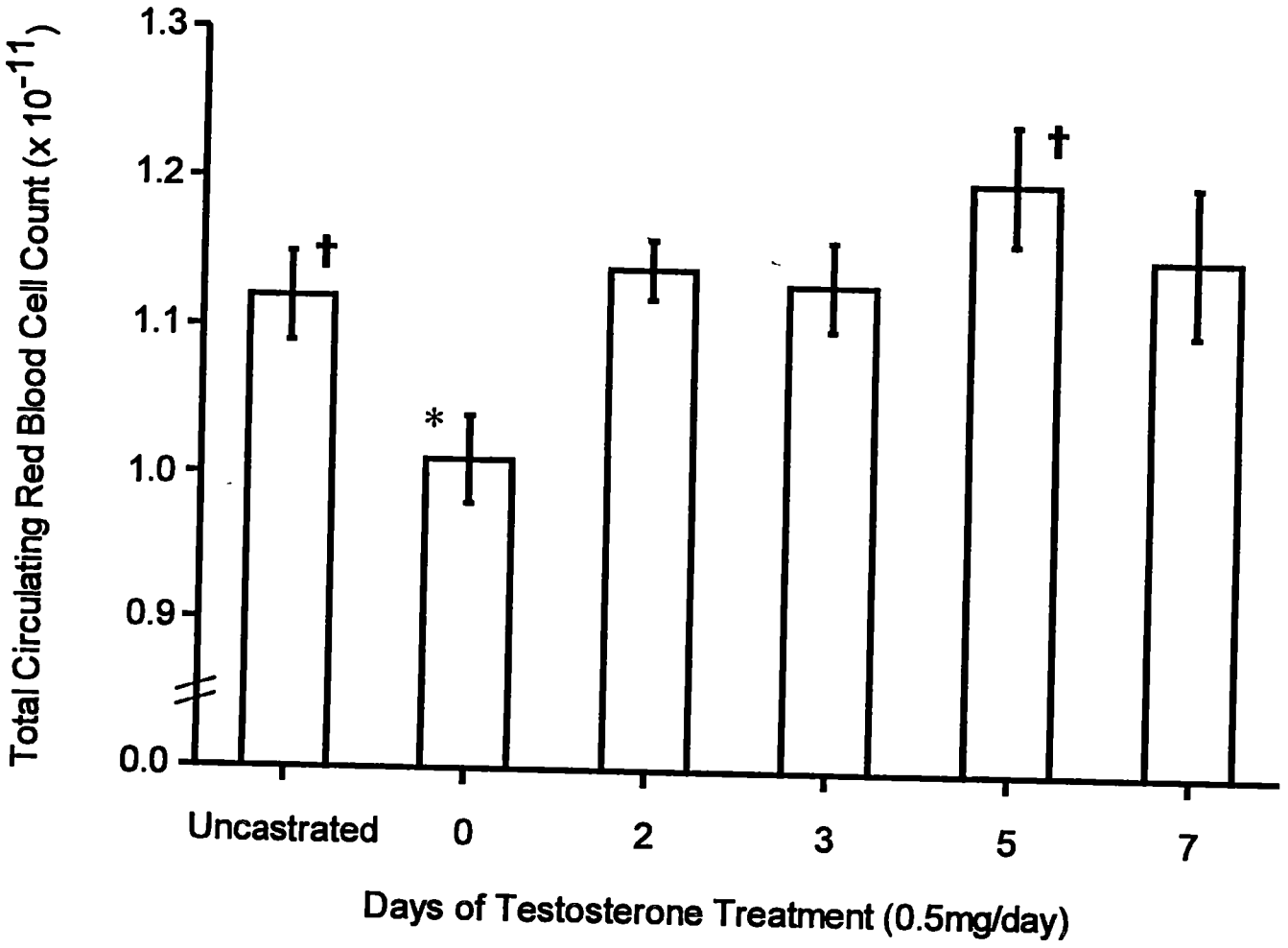
Total Circulating Red Blood Cell Count (x 10^{-11})
1.3
1.2
1.1
1.0
0.9
0.0
†
*
†
Uncastrated
0
2
3
5
7
Days of Testosterone Treatment (0.5mg/day)

Figure 4

Figure 5

The effects of castration and testosterone therapy on the reticulocyte counts of male BALB/c mice Means are indicated by wide vertical bars, with one S E indicated by the smaller bar. Means represent a total of 20, 25, 5, 10, 14, and 10 mice for treatments uncastrated, 0 (castrated), and 2,3,5 and 7 days of testosterone therapy, respectively Significance ($P <$ 0 05) is indicated by the following symbols *, significantly different from mean for uncastrated mice by Dunnett's t-tests, †, significantly different from mean for castrated mice receiving no testosterone therapy by Dunnett's t-tests

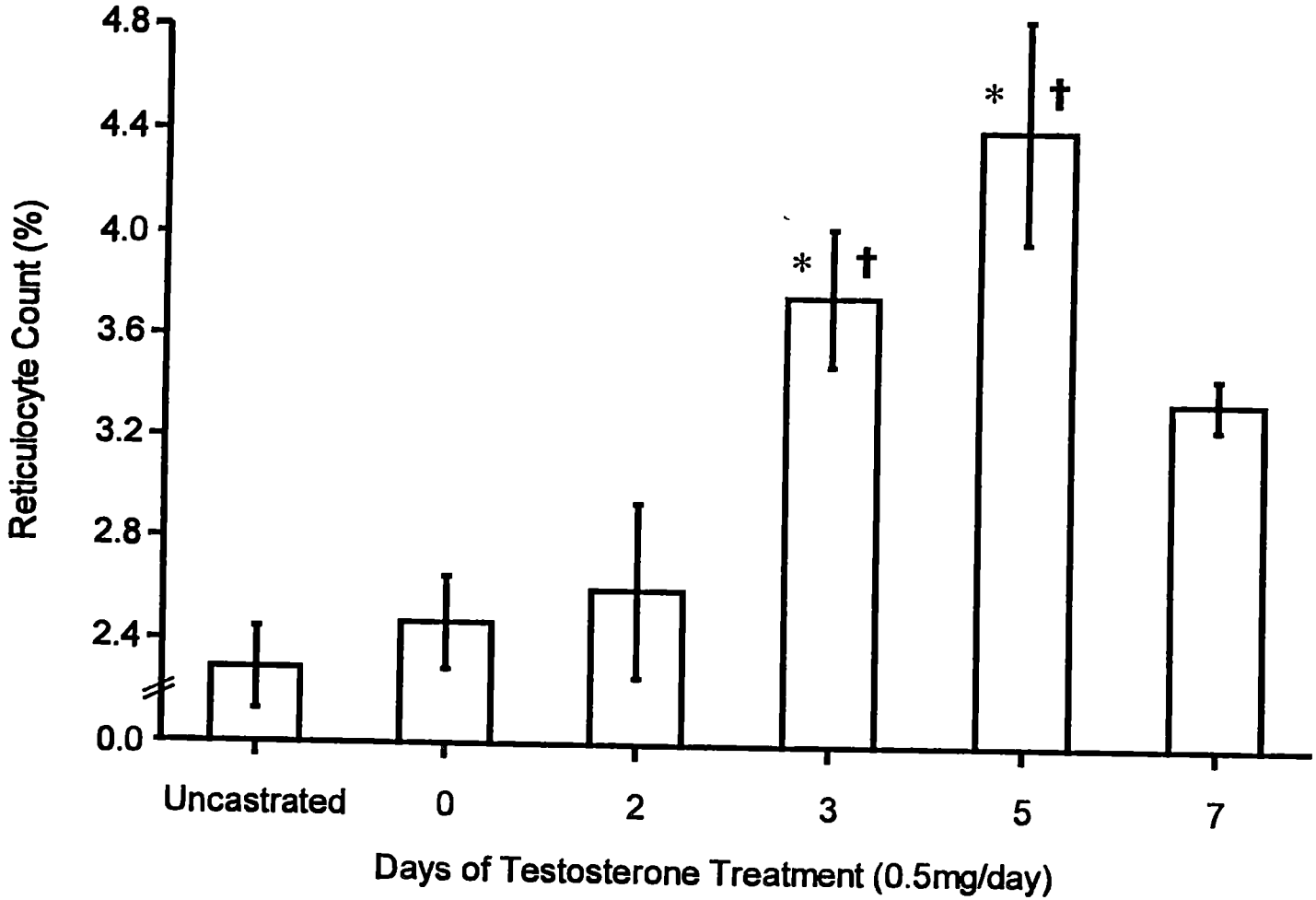
4.8
4.4
4.0
3.6
3.2
2.8
2.4
0.0
Reticulocyte Count (%)
*
†
*
†
Uncastrated
0
2
3
5
7
Days of Testosterone Treatment (0.5mg/day)

Figure 5

Figure 6

The effects of castration and testosterone therapy on the platelet counts of male BALB/c mice Means are indicated by wide vertical bars, with one S E indicated by the smaller bar Means represent a total of 25, 36, 18, 10, 14, and 20 mice for treatments uncastrated, 0 (castrated), and 2,3,5 and 7 days of testosterone therapy, respectively Significance ($P <$ 0 05) is indicated by the following symbols *, significantly different from mean for uncastrated mice by Dunnett's t-tests, †, significantly different from mean for castrated mice receiving no testosterone therapy by Dunnett's t-tests

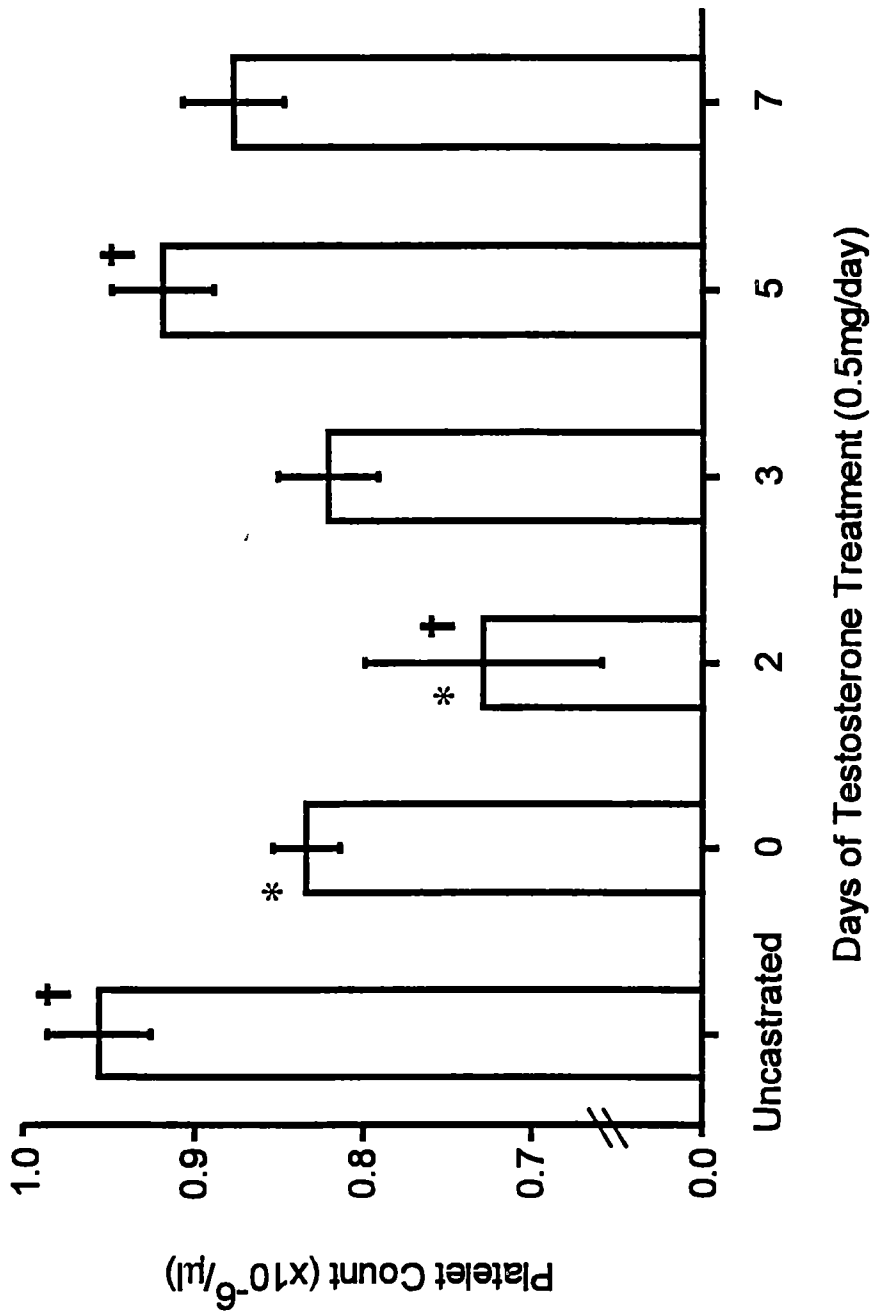
Platelet Count (x10^{-6}/μl)
1.0
0.9
0.8
0.7
0.0
Uncastrated
0
2
3
5
7
Days of Testosterone Treatment (0.5mg/day)
*
*
†
†
†

Figure 6

Figure 7

The effects of castration and testosterone therapy on the mean platelet sizes of male BALB/c mice. Means are indicated by wide vertical bars, with one S E indicated by the smaller bar Means represent a total of 23, 29, 10, 9, 12, and 11 mice for treatments uncastrated, 0 (castrated), and 2,3,5 and 7 days of testosterone therapy, respectively Significance ($P <$ 0 05) is indicated by the following symbols *, significantly different from mean for uncastrated mice by Dunnett's t-tests, †, significantly different from mean for castrated mice receiving no testosterone therapy by Dunnett's t-tests

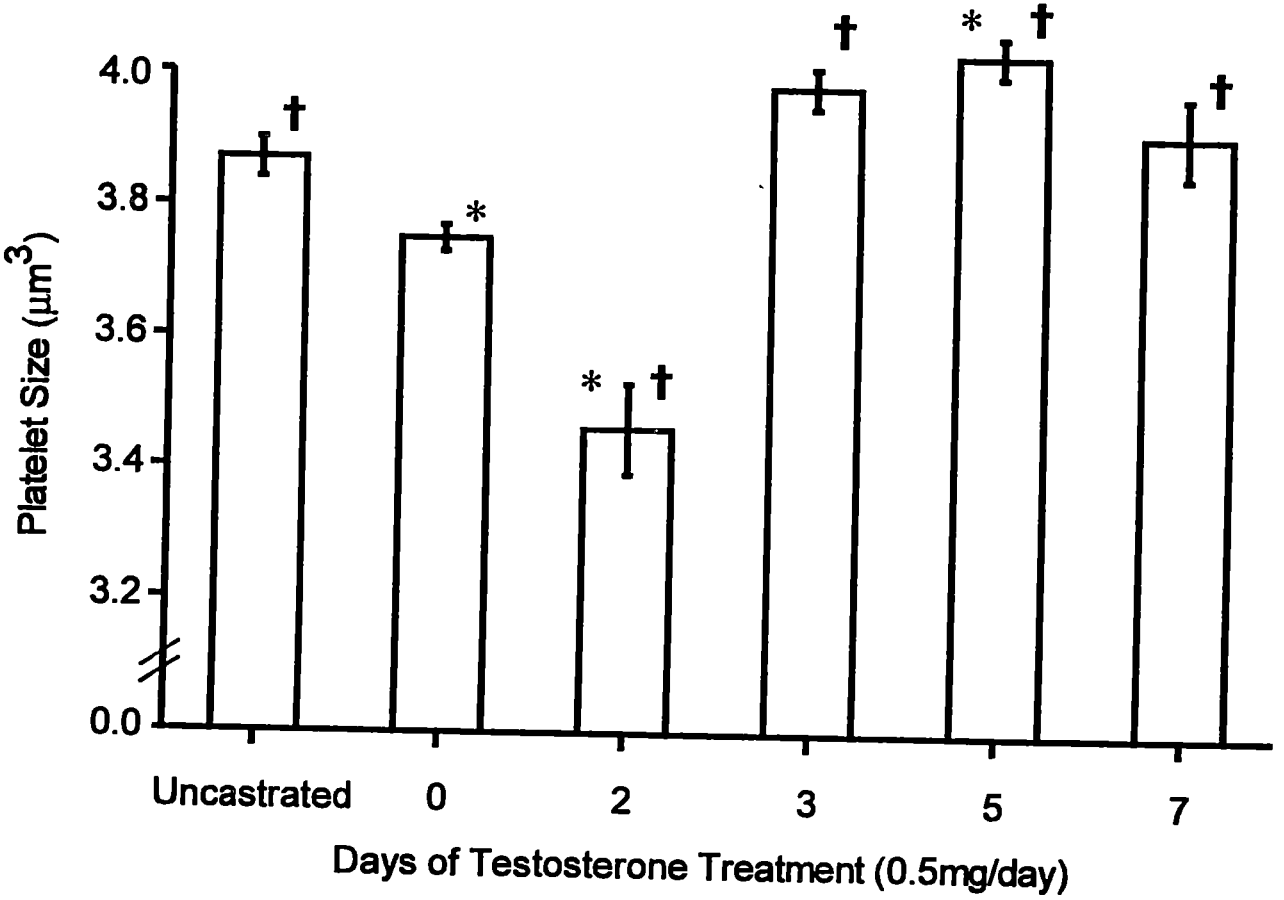
Platelet Size (μm3)
4.0
3.8
3.6
3.4
3.2
0.0
Uncastrated
0
2
3
5
7
Days of Testosterone Treatment (0.5mg/day)

Figure 7

Figure 8

The effects of castration and testosterone therapy on the $\%^{35}S$ incorporation into platelets of male BALB/c mice Means are indicated by wide vertical bars, with one S E indicated by the smaller bar Means represent a total of 23, 35, 17, 10, 14, and 20 mice for treatments uncastrated, 0 (castrated), and 2,3,5 and 7 days of testosterone therapy, respectively Significance ($P < 0\ 05$) is indicated by the following symbols *, significantly different from mean for uncastrated mice by Dunnett's t-tests, †, significantly different from mean for castrated mice receiving no testosterone therapy by Dunnett's t-tests

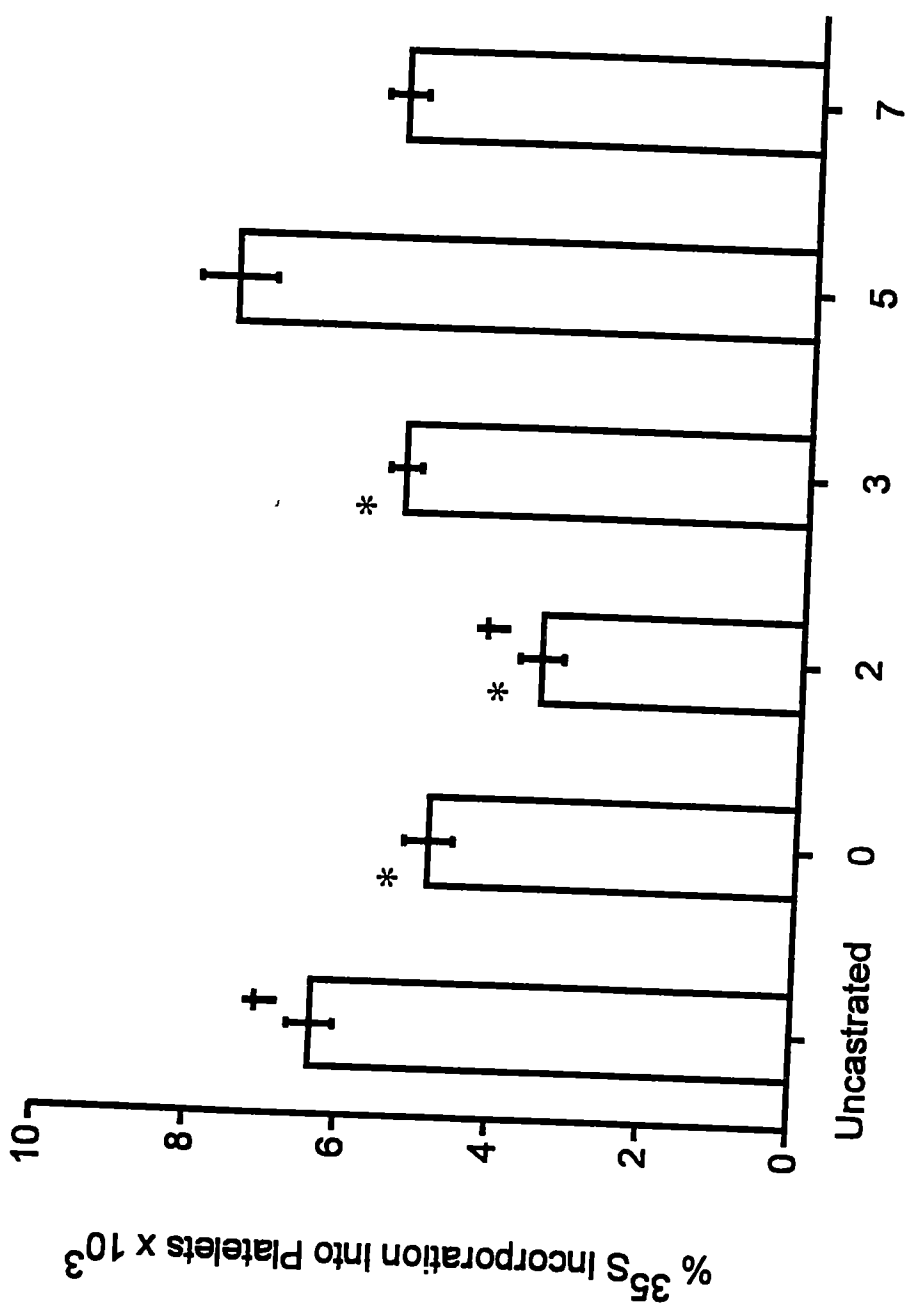
% 35S Incorporation into Platelets x 10^3
10
8
6
4
2
0
Uncastrated
0
2
3
5
7
Days of Testosterone Treatment (0.5mg/day)
*
*
*

Figure 8

Figure 9

The effects of castration and testosterone therapy on the total circulating platelet counts (TCPC) of male BALB/c mice Means are indicated by wide vertical bars, with one S E. indicated by the smaller bar Means represent a total of 25, 36, 18, 10, 14, and 10 mice for treatments uncastrated, 0 (castrated), and 2,3,5 and 7 days of testosterone therapy, respectively Significance ($P < 0\ 05$) is indicated by the following symbols *, significantly different from mean for uncastrated mice by Dunnett's t-tests, †, significantly different from mean for castrated mice receiving no testosterone therapy by Dunnett's t-tests

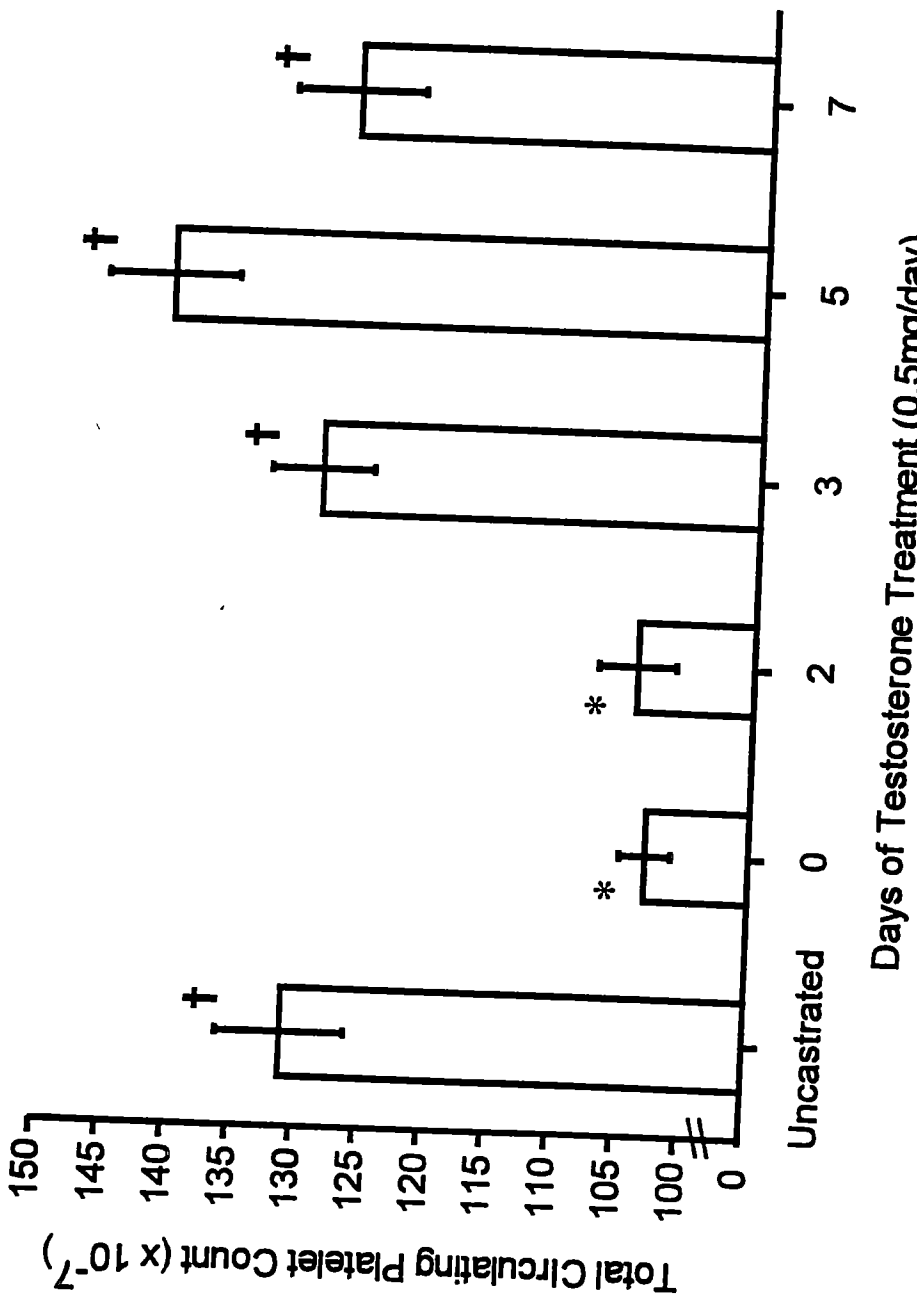
Total Circulating Platelet Count (x 10^7)
150
145
140
135
130
125
120
115
110
105
100
0
Uncastrated
0
2
3
5
7
Days of Testosterone Treatment (0.5mg/day)
*
*
+
+
+
+

Figure 9

Figure 10

The effects of castration and testosterone therapy on the total circulating platelet mass (TCPM) of male BALB/c mice Means are indicated by wide vertical bars, with one S E indicated by the smaller bar Means represent a total of 23, 29, 10, 9, 14, and 11 mice for treatments uncastrated, 0 (castrated), and 2,3,5 and 7 days of testosterone therapy, respectively Significance ($P < 0\ 05$) is indicated by the following symbols *, significantly different from mean for uncastrated mice by Dunnett's t-tests, †, significantly different from mean for castrated mice receiving no testosterone therapy by Dunnett's t-tests

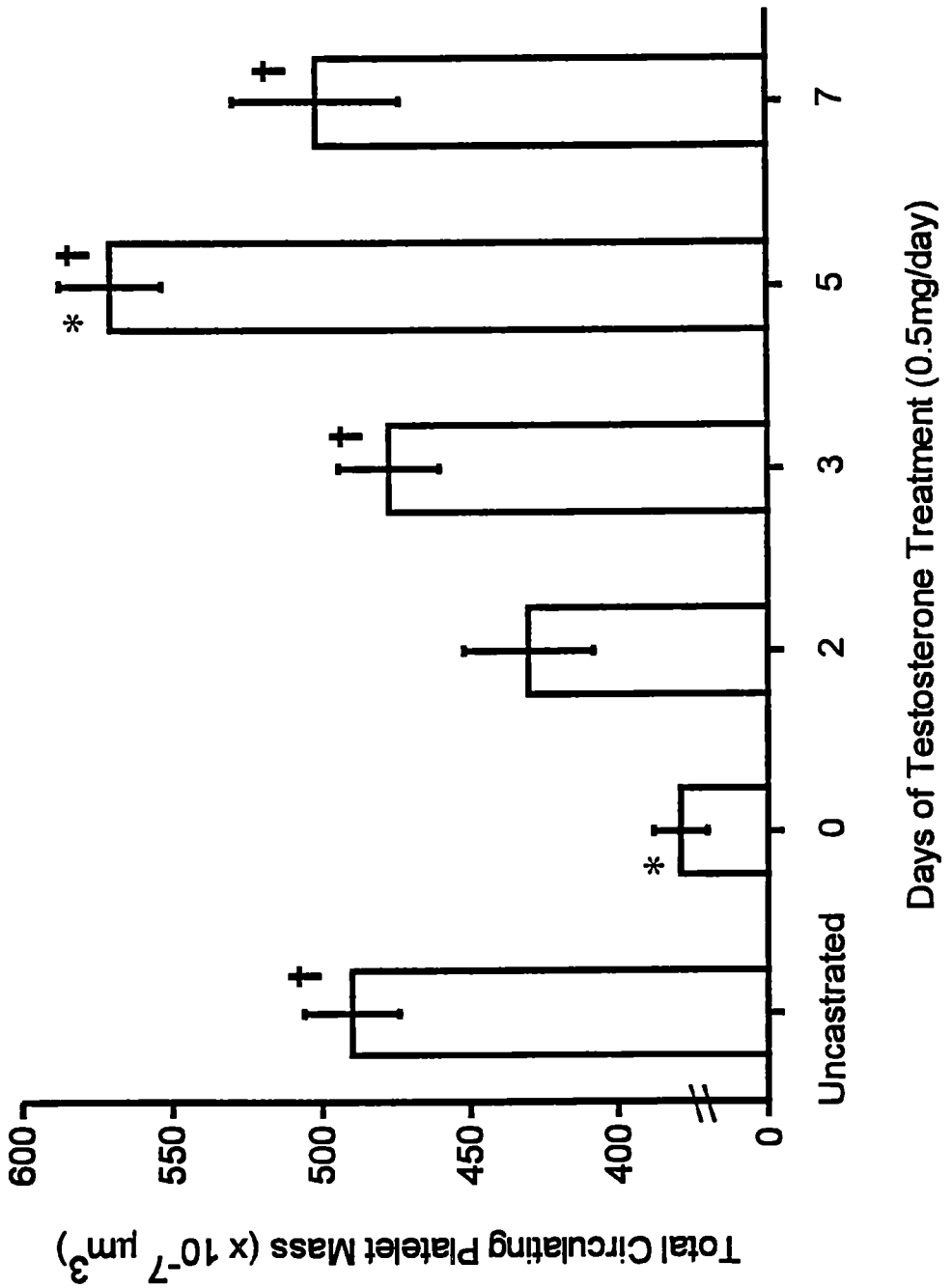
Total Circulating Platelet Mass (x 10^-7 µm^3)
600
550
500
450
400
0
Uncastrated
0
2
3
5
7
Days of Testosterone Treatment (0.5mg/day)
*
*
+
+
+
+

Figure 10

Figure 11

The effects of castration and testosterone therapy on the mean megakaryocyte ploidy of male BALB/c mice Means are indicated by wide vertical bars, with one S E indicated by the smaller bar Means represent a total of 21, 25, 12, 8, 14, and 10 mice for treatments uncastrated, 0 (castrated), and 2,3,5 and 7 days of testosterone therapy, respectively Significance ($P < 0\ 05$) is indicated by the following symbols *, significantly different from mean for uncastrated mice by Dunnett's t-tests, †, significantly different from mean for castrated mice receiving no testosterone therapy by Dunnett's t-tests

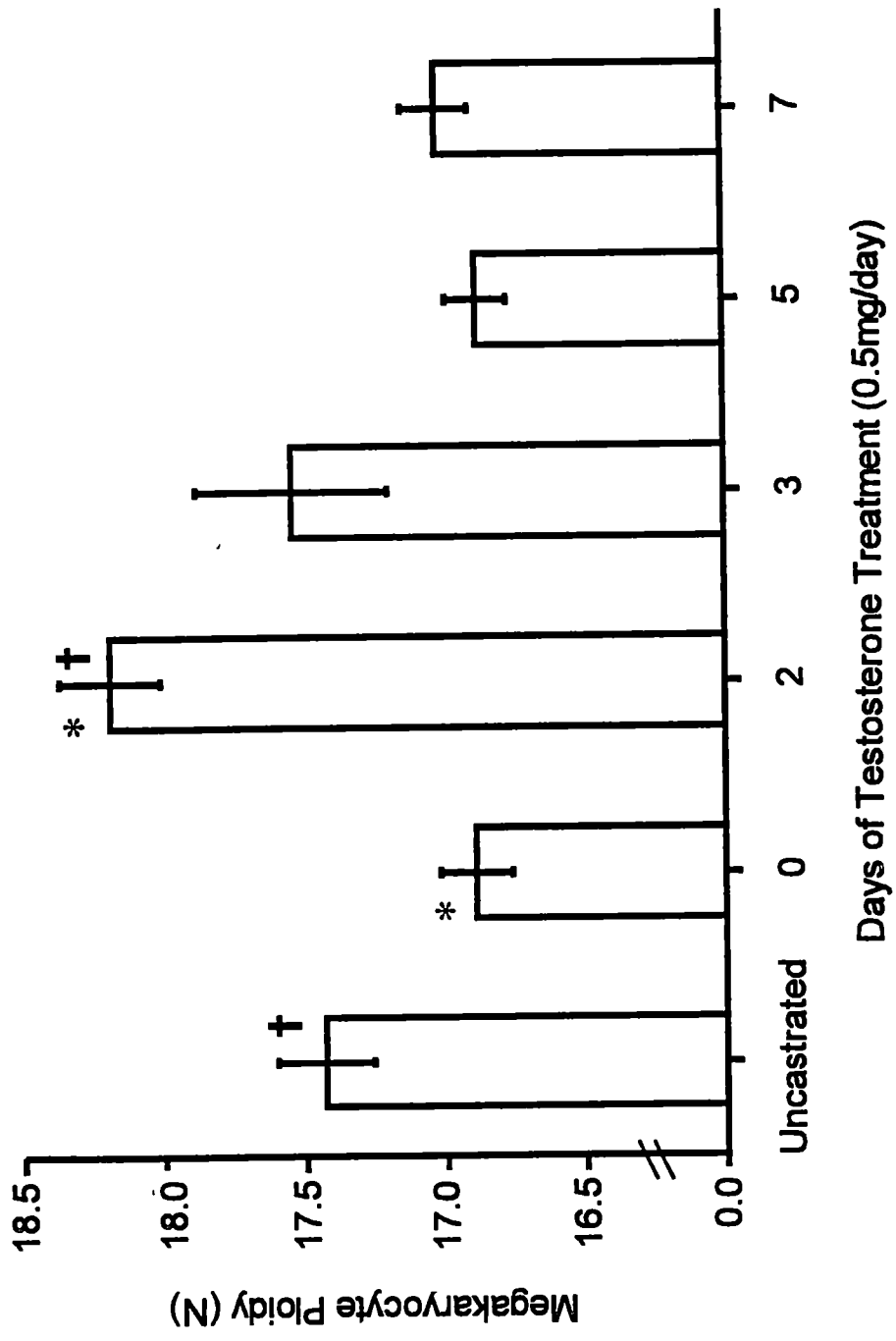
Megakaryocyte Ploidy (N)
18.5
18.0
17.5
17.0
16.5
0.0
Uncastrated
0
2
3
5
7
Days of Testosterone Treatment (0.5mg/day)
*
*
+
+

Figure 11

Figure 12

The effects of castration and testosterone therapy on white blood cell (WBC) counts of male BALB/c mice Means are indicated by wide vertical bars, with one S E indicated by the smaller bar Means represent a total of 25, 36, 18, 10, 14, and 20 mice for treatments uncastrated, 0 (castrated), and 2,3,5 and 7 days of testosterone therapy, respectively Significance ($P < 0\ 05$) is indicated by the following symbols *, significantly different from mean for uncastrated mice by Dunnett's t-tests, †, significantly different from mean for castrated mice receiving no testosterone therapy by Dunnett's t-tests

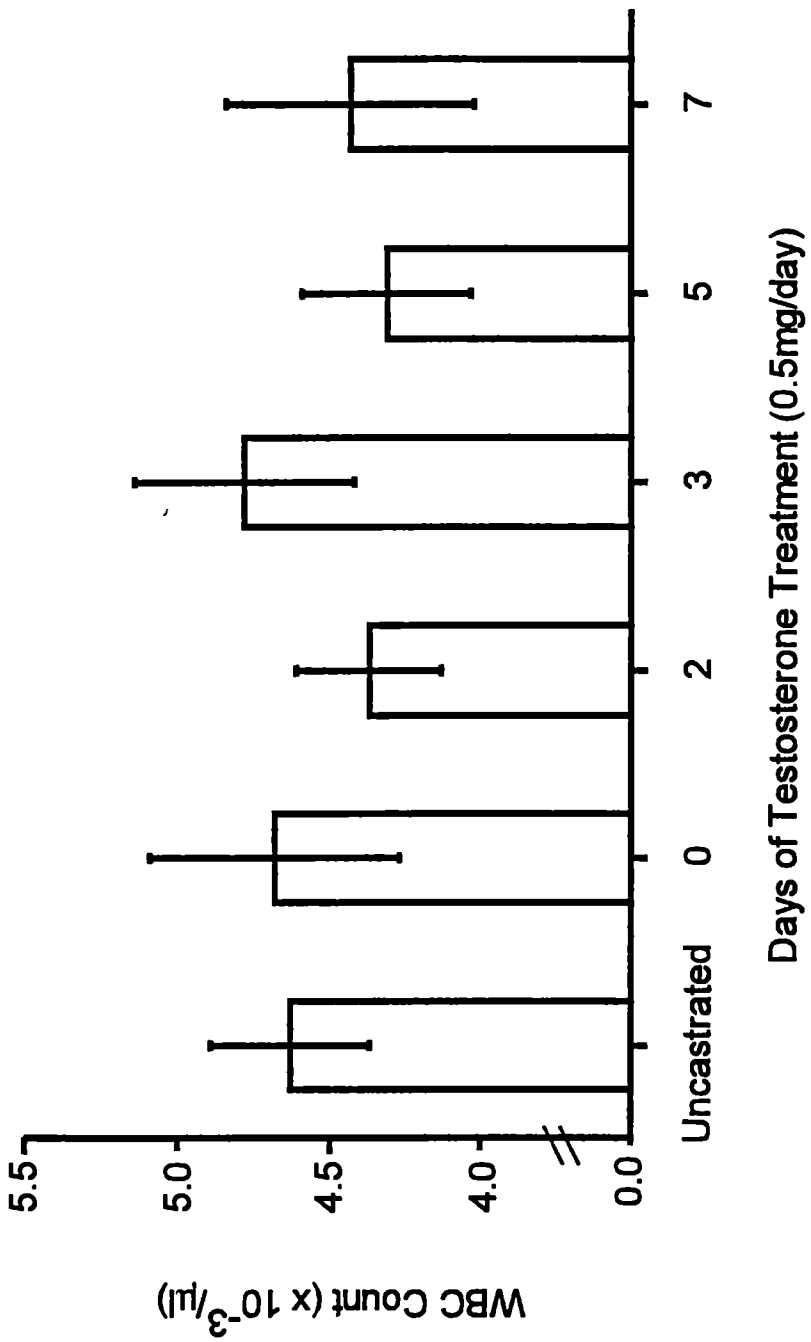
WBC Count (x 10^-3/μl)
5.5
5.0
4.5
4.0
0.0
Uncastrated
0
2
3
5
7
Days of Testosterone Treatment (0.5mg/day)

Figure 12

Figure 13

A commentary on the restriction of colony forming cells (CFC) originally proposed by Nicola and Johnson Abbreviations for cells in the scheme are as follows CFU-S, colony forming unit - spleen, Mϕ, macrophage, EO, eosinophil, GM, granulocyte-macrophage, G, granulocyte (i e neutrophil), Meg, megakaryocyte, E, erythroid The proposed site of action of testosterone is indicated by the arrow and shaded cell Redrawn with commentary and modification from reference [24]

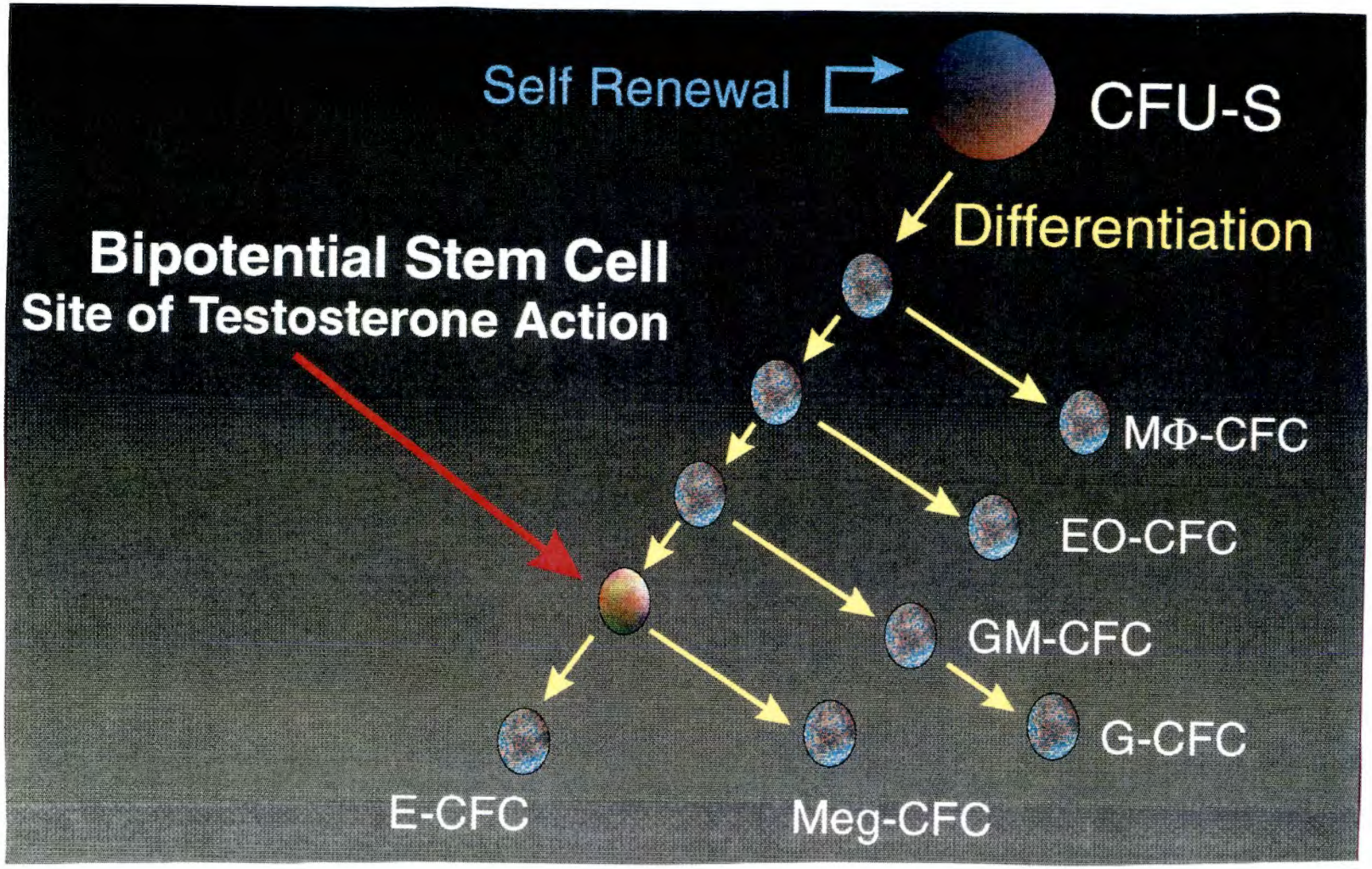
Self Renewal
CFU-S
Differentiation
Bipotential Stem Cell
Site of Testosterone Action
MΦ-CFC
EO-CFC
GM-CFC
G-CFC
E-CFC
Meg-CFC

Figure 13

Table I

Linear regression of the effects on red blood cell (RBC) indices of the administration of testosterone to castrated male BALB/c mice Castration is considered as the 0 time point, with other treatments represented as number of days Total number of mice in the regression analysis was 98, 64, 58, 53 and 53 for PCV, reticulocyte count, total circulating red blood cell count (TCRBCC) and total circulating red blood cell mass (TCRBCM), respectively

Table I

Parameter	F-value	*P* Value	Intercept	Slope
PCV	7 2	0 009	47 3	0 2
Reticulocytes	21 1	<0 0001	2 54	0 3
TCRBCC	5 6	0 02	1029	16
TCRBCM	108	<0 0001	6 03	0 21

Table II

Linear regression of the effects on platelet indices of the administration of testosterone to castrated male BALB/c mice Castration is considered as the 0 time point, with other treatments represented as number of days Total number of mice in the regression analysis was 98, 98, 98, 71 and 96 for platelet count, platelet size, total circulating platelet count (TCPC), and total circulating platelet mass (TCPM), and $\%^{35}S$ incorporation into platelets, respectively

Table II

Parameter	F-value	*P* Value	Intercept	Slope
Platelet Count	6 0	0 02	0 804	0 01
Platelet Size	14 3	0 0003	3 82	0 03
TCPC	45 2	< 0 0001	103 3	4 4
TCPM	54 6	< 0 0001	388 9	23 6
$\%^{35}S$ incorporation into platelets	9 6	0 002	4 58	0 21

Part 4

Platelet Production in Hypothyroid Dogs and Hyperthyroid Cats

Chapter 1

Abstract

Changes in platelet indices (platelet count and platelet size) and PCV associated with thyroid disease were studied in 7 dogs with hypothyroidism and 21 cats with hyperthyroidism admitted to the veterinary teaching hospital Compared with control (euthyroid) animals, dogs with hypothyroidism had higher platelet count (P=0 003), smaller platelet size (P=0 01), and lower PCV (P=0 02) Comparison of the group of hyperthyroid cats with a group of similarly aged, clinically normal cats with normal thyroxine values indicated that the group of hyperthyroid cats had significantly (P=0 03) higher mean platelet size than did control cats, but differences were not found in mean platelet count or PCV The results of this investigation indicate that the changes in platelet size reported in human beings with thyroid endocrinopathies are also found in animals affected with the analogous thyroid disease Although the pathogenesis of platelet abnormalities in animals with thyroid derangement is unclear and likely is multifactorial, the observed relation between platelet and erythrocyte production in this group of dogs is consistent with reports of an inverse relation between thrombocytopoiesis and erythropoiesis in iatrogenically hyperthyroid mice and in mice exposed to hypoxia

Chapter 2

Introduction

The actions of thyroid hormones on erythrocyte production are well defined Results of *in vitro* [1,2] and *in vivo* [3] studies have indicated that thyroid hormones stimulate erythropoiesis These results are in agreement with the clinical findings in thyroid deranged animals. in one review [4], about 20% of cats with hyperfunctional thyroid adenomas had erythrocytosis, and in a study of 56 dogs with hypothyroidism [5], normocytic, normochromic anemia was observed in about half of the subjects In human beings with hyperthyroidism, an increase in PCV and in blood volume has been reported [6] Also, up to 60% of human beings with hypothyroidism are anemic [6] It is well established that the mechanisms of thyroxine's erythroid stimulation are direct, via a β_2 adrenergic receptor [2], and indirect, via increased cellular oxygen demands and resulting release and action of erythropoietin [1] Another infrequent cause of altered erythrocyte production in human beings with thyroid disease is autoimmune hemolytic anemia, which may develop in people with either Graves' disease (hyperthyroidism) or Hashimoto's thyroiditis (hypothyroidism) [7]

Panzer et al [8] recently suggested that the thrombocytopenia and other changes in platelet indices seen in association with hyperthyroidism are secondary to an unspecified effect of excess thyroxine concentration (e g , a metabolic effect), and may not always be attributable to a concurrent or related autoimmune process In support of this hypothesis, recent data from our laboratory indicated that experimentally-induced hyperthyroidism in mice

resulted in stimulation of erythropoiesis with concurrent decrease of platelet production [9].

Most cases of hyperthyroidism in cats are attributable to hyperfunctional adenomas (adenomatous hyperplasia) [4] Recently, evidence of an autoimmune mechanism of hyperthyroidism was reported in some cats with hyperthyroidism [10] It is possible that induction of an autoimmune response may occur after retroviral infection, as retroviruses have been implicated in human autoimmune thyroid disease [11]

In dogs, the most common causes of hypothyroidism are lymphocytic thyroiditis and idiopathic atrophy [5] Mechanisms of thyroiditis are often autoimmune in nature, with antibodies to at least one thyroid moiety (thyroglobulin, T_4, or T_3) found in roughly half of the dogs with hypothyroidism [12] It has been observed [13] that hypothyroidism may exacerbate hemostatic defects in clinically normal canine carriers of von Willebrand's disease, and that hormonal abnormalities, including hypothyroidism, may be predisposing factors to development of autoimmune blood diseases [14]

The purpose of the study reported here was to evaluate alterations in platelet indices in dogs and cats with thyroid diseases

Chapter 3

Materials and Methods

Study groups The 7 dogs included in the hypothyroid group were referred to the dermatology service at the veterinary teaching hospital, where the diagnosis of hypothyroidism was made on the basis of results of a thyroid releasing hormone (TRH) stimulation test [15] Blood for analysis of platelet indices was collected before thyroid hormone replacement therapy was initiated Mean age of the dogs was 6 7 years (range, 3 to 12 years), and breeds represented were (1 each) Shih Tzu, Labrador Retriever, Golden Retriever, Schnauzer, Boxer, Cocker Spaniel, and mixed breed (14% each) Of the dogs, 3 of 7 (42%) were spayed females, 2 (29%) were sexually intact females, and 2 (29%) were sexually intact males Control dogs also were referred to the dermatology service because of pruritus, and were proven euthyroid by results of a TRH stimulation test [15] Of the 11 control dogs, 3 (27%) were spayed females, 3 (27%) were castrated males, 2 (18%) were sexually intact females, and 2 (18%) were sexually intact males Mean age of the control dogs was 5 9 years (range, 1 to 11 years), breeds represented were 1 (9%) each of English Setter, Flat Coated Retriever, Fox Terrier, and Swiss Mountain Dog, 2 (18%) each of Golden Retriever and Labrador Retriever, and 3 (27%) mixed-breed dogs

The 21 cats included in the hyperthyroid group were patients admitted for radioiodine (^{131}I) therapy for hyperthyroidism Mean age of the cats was 13 7 years (range, 7 to 16 years), and breeds represented were Domestic Short Hair (67%), Siamese (28%), and Persian

(5%) In all cases, a technetium 99 (^{99}Tc) scan was performed prior to therapy, and determination of the cause of hyperthyroidism was made on the basis of ^{99}Tc uptake patterns or, in 1 cat, biopsy findings of the affected gland Some cats had been previously treated for hyperthyroidism using methimazole, but in each cat, administration of the drug had been discontinued for at least 14 days prior to collection of blood samples for platelet analysis

Controls (n=10) were clinically normal cats volunteered by owners for participation in the study Of the 10 cats, 2 (20%) were spayed females, 2 (20%) were sexually intact females, 4 (40%) were castrated males, and 2 (20%) were sexually intact males Mean age of control cats was 12 1 years (range, 6 to 17 years) Breeds represented were Siamese (1 of 10) and Domestic Short Hair (9 of 10) Incidental problems observed in the control cats at the time of physical examination (1 cat each) included chronic ocular and nasal discharge, a II/VI holosystolic heart murmur, palpably small kidneys, and feline acne and flea allergy dermatitis

Blood sample collection and platelet analysis Blood samples for platelet size analysis were collected into plastic syringes by single jugular venipuncture and were quickly transferred into evacuated collection tubes (Vacutainer, Becton Dickinson, Rutherford, NJ) containing sodium citrate Blood samples were centrifuged at 22°C for 4 5 minutes at 160 x g to allow preparation of platelet-rich plasma (PRP) A small aliquot of the PRP was diluted in buffered isotonic saline solution (Isoton, Baxter Healthcare Corp, McGaw Park, IL) and subjected to platelet size analysis, using an instrument (Electrozone Celloscope, Particle Data Inc, Elmhurst, IL) equipped with a 128-multichannel analyzer [16] The instrument was calibrated at frequent intervals using 2 02 μm diameter latex particles

Platelet size is reported as mean volume after normalization of the data Reference ranges at our laboratory for mean platelet size are 8 to 12 μm^3 (canine) and 7 to 14 μm^3 (feline)

Blood samples for platelet count and PCV determinations were collected via jugular venipuncture and immediately transferred into evacuated collection tubes containing sodium EDTA (Vacutainer, Becton Dickinson, Rutherford, NJ) Platelet count was performed manually, using a hemacytometer chamber and phase microscopy, and PCV was determined using a standard microtechnique Reference ranges for our laboratory are platelet count 1 5 to 4 0 x 10^5 platelets/µl (canine) and 1 7 to 4 5 x 10^5 platelets/µl (feline), PCV 36 to 55% (canine), and 26 to 45% (feline)

Thyroxine determination. Baseline total thyroxine (T_4) concentration was determined in control cats and in 16 of the hyperthyroid group cats by use of radioimmunoassay with a commercially available kit (Diagnostic Products Corporation, Los Angeles, CA) Reference values for the veterinary teaching hospital laboratory are 10 to 40 ng/µl. In the other 5 cats, T_4 concentration was determined by independent veterinary reference laboratories For all cats, values were converted to a standard unit -nanograms per milliliter In the hypothyroid dogs, the TRH stimulation test [15] was performed by measuring T_4 concentration before and 6 hours after i v administration of TRH (Sigma Chemical Co, St Louis, MO) at dosage of 0.05 mg/kg of body weight (maximal dose 1 0 mg) To be considered euthyroid, a dog must achieve a 1 5-fold increase in T_4 concentration 6 hours after TRH stimulation, and the T_4 concentration at that time must exceed 25 ng/ml

Statistical analysis Statistical analysis of data was performed by use of the Students *t*-test and statistical software (PROC T TEST, SAS Institute, Cary, NC) A P value < 0 05

was considered to be statistically significant in a two-tailed test

Chapter 4

Results

Hypothyroid dogs Of the 7 hypothyroid dogs, 4 (57%) were anemic, and 2 (29%) had thrombocytosis (platelet count > 400,000/μl) Compared with values in euthyroid control dogs (Figure 1), platelet count was significantly (P=0 003) higher (mean platelet count, 361 x 10^3/μl vs 200 x 10^3/μl) and PCV was significantly (P=0 02) lower (mean PCV 36 9% vs 45 5%) Mean platelet size was significantly (P=0 01) lower than that in euthyroid controls (mean, 8 00 μm^3 vs 10 08 μm^3)

Hyperthyroid cats Of the 21 hyperthyroid cats, 3 (14%) were thrombocytopenic, and 1 (5%) had erythrocytosis. Of the 21 cats, 19 (90%) were hyperthyroid because of hyperfunctional thyroid adenomas (adenomatous hyperplasia), and the remaining 2 had hyperthyroidism attributed to thyroid carcinoma In comparison with the 10 clinically normal (control) cats with normal T_4 concentrations (Figure 2), cats in the study group had significantly (P=0 03) larger mean platelet size (mean size 13 11 μm^3 vs 10 93 μm^3), and significantly higher T_4 concentrations (data not shown) The difference in platelet count or PCV between the 2 groups was not statistically significant

Chapter 5

Discussion

The effects of thyroxine on platelet production are not well characterized Much of the data on this subject has come in the form of case reports in human medicine Clinically recognized thrombocytopenia is infrequent in hyperthyroid people, but even in those without frank thrombocytopenia, an increase in bone marrow megakaryocytes [17] and a decrease in platelet survival time [18] have been reported Although Lamberg et al [19] described thrombocytopenia in people with Graves' disease, Endo [20] reported that thyroid hormone concentrations and platelet count are not predictably correlated Kurata et al [18] proposed 3 mechanisms for the changes in platelet count and survival time autoimmune (i e , a common autoimmune mechanism for Graves' disease and thrombocytopenia), direct damage to platelets by thyroid hormones, and decreased platelet life span secondary to monocyte/macrophage (reticuloendothelial) system activation (supported in human beings by a common clinical finding of splenomegaly) High platelet-bound IgG concentration has been observed in thrombocytopenic patients with Graves' disease and Hashimoto's thyroiditis, supporting an autoimmune effect on platelets associated with both diseases [21] However, high IgG concentration was also found in patients with normal platelet counts [21] Conversely, marked thrombocytopenia can be associated with negative platelet antibody findings [22] In summary, platelet count in human beings with autoimmune thyroid disease may be normal or low, and platelet count has inconsistent association with amounts of platelet

associated IgG

Human thyroid disease is very complex in its pathogenesis For example, patients may have concurrent Graves' disease and Hashimoto's thyroiditis [23] Another population have a spontaneously resolving hyperthyroid period associated with subacute thyroiditis [23] The underlying theme in human thyroid disease is autoimmunity [24,25]

Recently, Ford et al [26] reported increased mean platelet volume in human hyperthyroid patients, while van Doormaal et al [27] have observed a decreased platelet size in human hypothyroid patients Again, several possible explanations of these changes were presented, including an auto-immune mechanism

Our findings in these thyroid deranged animals were generally in agreement with previously reported data from human beings with thyroid disease In agreement with previous reports in human beings, [26,27] we observed increased mean platelet size in hyperthyroid cats, and anemia and decreased mean platelet size in hypothyroid dogs Additionally, we report an increase in mean platelet count in hypothyroid dogs, compared with euthyroid dogs Although it was reported that breed predilection for hyperthyroidism in cats does not exist [28], Siamese cats were over-represented in our hyperthyroid group (28% of hyperthyroid cats, compared with 6% of total feline admissions to the veterinary teaching hospital during the same period) This finding could reflect selection bias, because owners of a purebred cat may be more likely to pursue ^{131}I treatment for hyperthyroidism (the basis for inclusion in this study) Alternatively, Siamese cats may be more likely to have hyperthyroidism Additionally, we noticed that our reference range for platelet size in cats is lower than that reported by Weiser and Kociba [29] This difference may be attributable to choice of anti-

coagulant, as citrate (used in our study) is known to cause less swelling of canine platelets than does EDTA [30], or to different centrifugation techniques used for preparation of platelet-rich plasma

Perhaps the most interesting aspect of our findings is the comparative evaluation of changes in platelet production in animals and human beings with thyroid disease Because the most hyperthyroidism in people is thought to be caused by an immune-mediated process (Graves' disease), an auto-immune mechanism for thrombocytopenia and increased platelet size has been suggested [7,18] We report similar changes in a group of cats in which the cause of hyperthyroidism was hyperfunctional adenoma in 90% of the cases This finding may be explained by the hypothesis of Panzer [8] that a metabolic mechanism accounts for the changes in thrombocytopoiesis seen in patients with thyroid disease

Of further interest is the inverse relation between platelet count and PCV in our hypothyroid dogs One mechanism that is consistent with these changes is that of progenitor-cell competition This theory purports that a common progenitor cell in the bone marrow can contribute to either the erythrocytic or megakaryocytic cell lines, and that strong stimulation of that cell line can result in decreased availability of immature precursor cells to the other There is considerable evidence in support of this mechanism, which has been documented not only in thyroxine treated mice [9], but also in mice subjected to hypoxia The work with hypoxia showed that long-term hypoxia increases erythrocytopoiesis while concurrently decreasing thrombocytopoiesis, as measured by platelet count [31-33], percentage of ^{35}S incorporation into platelets [31-34], colony-forming unit megakaryocyte populations [35], megakaryocyte precursor cell populations [36], and megakaryocyte concentration in the bone

marrow and spleen [37] Recent work in our laboratory indicated a similar inverse relation between thrombocytopoiesis and erythrocytopoiesis in mice with iatrogenic hyperthyroidism [9] Numerous biochemical and antigenic similarities exist between the 2 cell lines, providing further evidence in support of this model [38]

The aforementioned model would predict that in hypothyroid patients, inadequate concentration of T_4, a hormone that is known to stimulate erythropoiesis, should result in decreased production of erythrocytes and allow more of the common progenitor cell population to enter the megakaryocyte cell line, resulting in increased platelet production These predicted changes were observed in our hypothyroid dogs It seems reasonable that the decreased platelet size observed was related to the high platelet count in this group, because an inverse relation between platelet count and platelet size has been described [39] Alternatively, decreased platelet size in hypothyroid patients may indicate increased platelet survival time

Future work should include measurements of antithyroglobulin and T_3 and T_4 antibodies which could detect early immune mediated thyroid destruction that has not affected functional reserve [12,40] If a metabolic mechanism accounts for the changes in platelet production, as we propose, the effects are likely dose-dependent and such distinction is unlikely to be relevant Detection of early, non-clinical disease in study groups is more critical if an immune mechanism of altered platelet production is operative in these patients

This same progenitor cell competition model would predict thrombocytopenia and erythrocytosis in hyperthyroid cats, and although we observed these changes in individual hyperthyroid cats, mean platelet count or PCV was not different between the hyperthyroid

and euthyroid groups Das et al [41] reported that vitamin B_{12} and/or folate deficiency is a cause of normal or decreased erythrocyte production in hyperthyroid human beings In future clinical studies, serum concentration of vitamin B_{12}, folate, and iron should be evaluated to explore this possible confounding factor Presence of neoplasia in these cats may have affected platelet production or consumption, because neoplastic processes may alter platelet indices in many ways [42] Another possibility is that hyperthyroidism results in a change in platelet function, interruption of platelet function by administration of aspirin to mice has been shown to increase platelet size without changing platelet count [43] This possibility merits further investigation As well, in future work, evaluation of platelet-associated IgG concentration in animals with thyroid disease would be helpful in defining the role of autoimmunity in altered platelet indices Measuring platelet associated IgG concentration would also help to assess the possibility that early immune mediated thrombocytopenia could exist in the hypothyroid dogs, given the reported concurrent development of these diseases [14]

This study was intended to investigate changes in platelet production in thyroid-deranged animals, and to evaluate comparative aspects of these findings in reference to reports in human beings We report a highly significant and inverse relation between PCV and platelet count in hypothyroid dogs, compared with euthyroid dogs We propose that the progenitor cell competition model, described in experiments with hypoxia, is consistent with these changes. Further, we report that mean platelet size is increased in cats with hyperthyroidism as it is in human beings with Graves' disease. Future work in this field should involve measurement of platelet associated IgG concentration and platelet life span in dogs the

thyroid status of which has been characterized by measurement of thyroid auto-antibodies, and in hyperthyroid cats

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Appendix

Chapter 4

Figure 1

Platelet indices and PCV of 7 hypothyroid dogs and 11 euthyroid dogs Mean value is indicated by the horizontal bar The *P*-value for the euthyroid vs hypothyroid *t*-test appears above each column

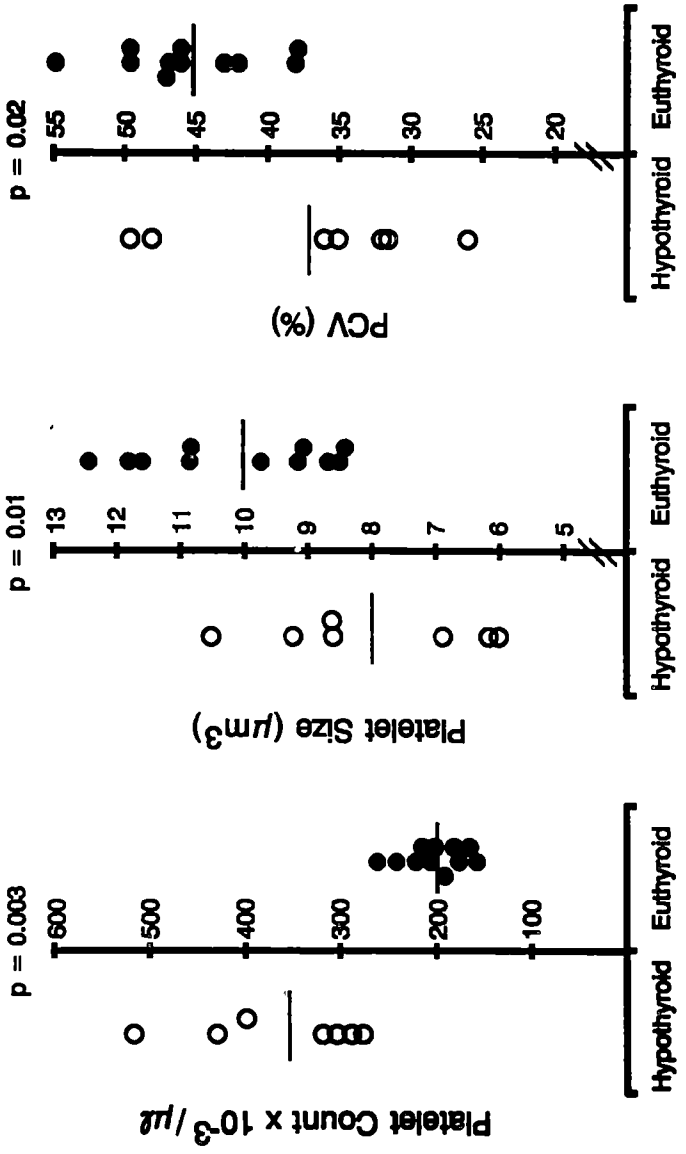
Platelet Count x $10^{-3}/\mu l$
p = 0.003
600
500
400
300
200
100
Hypothyroid
Euthyroid
Platelet Size (μm^3)
p = 0.01
13
12
11
10
9
8
7
6
5
Hypothyroid
Euthyroid
PCV (%)
p = 0.02
55
50
45
40
35
30
25
20
Hypothyroid
Euthyroid

Figure 1

Figure 2

Platelet indices and PCV of 21 hyperthyroid cats and 10 euthyroid cats The *P* value for difference in platelet size appears above the size graph NS = no statistical significance

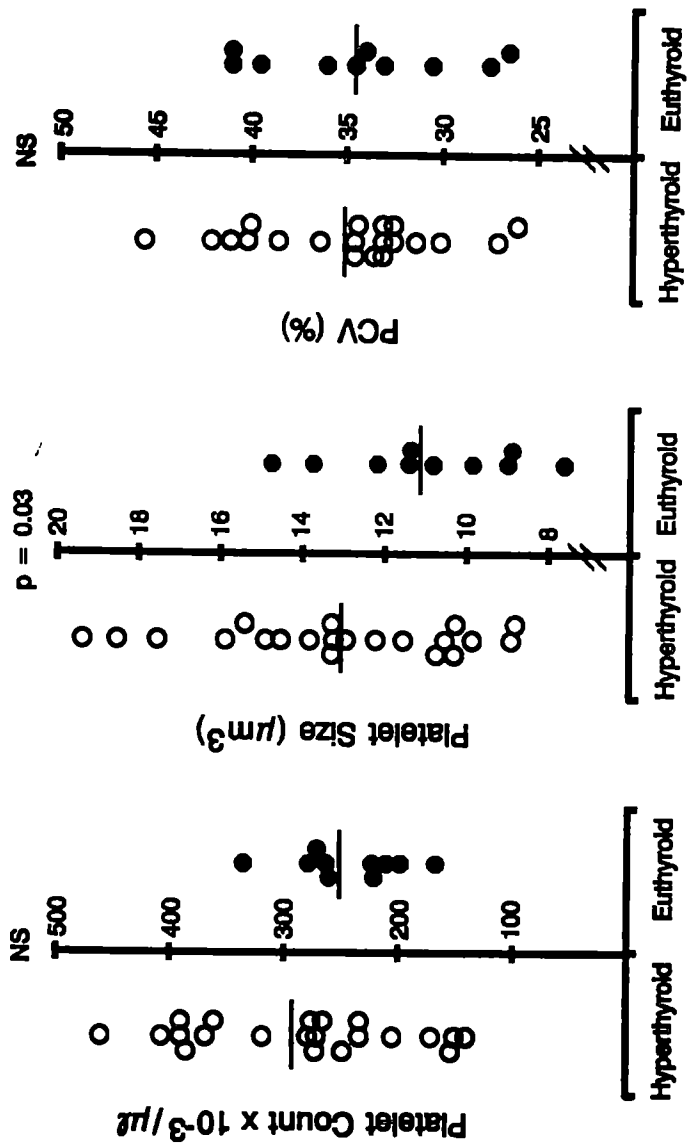
Platelet Count x 10-3/μl
NS
500
400
300
200
100
Hyperthyroid
Euthyroid
Platelet Size (μm3)
p = 0.03
20
18
16
14
12
10
8
Hyperthyroid
Euthyroid
PCV (%)
NS
50
45
40
35
30
25
Hyperthyroid
Euthyroid

Figure 2

Part 5

Hematologic Characteristics of Greyhound Dogs

Chapter 1

Abstract

A group of 36 greyhound dogs, from breeding farms in the Southeast and privately owned pets adopted through greyhound "rescue" programs, was used to study the hematologic characteristics in greyhounds A control group of 22 non-greyhound dogs, consisting of both animals housed in research facilities at the College of Veterinary Medicine and privately owned pets, served as control animals A representative group of greyhounds had no serologic evidence of exposure to *Ehrlichia canis* or *Babesia canis* Major findings in the erythron of greyhounds compared to control (non-greyhound) dogs included higher hemoglobin concentrations (mean 19 86 g/dl, $P < 0\ 0001$), higher packed cell volumes (mean 53 6%, $P < 0\ 0001$), higher mean corpuscular volume (mean 81 0 μm^3, $P = 0\ 0001$), higher mean cellular hemoglobin (mean 30 03 pg/cell, $P = 0\ 0001$), lower RBC counts (mean 6 66 x $10^6/\mu l$, $P = 0\ 003$), lower hemoglobin P_{50} (mean 23.5 mm O_2, $P < 0\ 0001$), and lower Hill coefficients (mean = 1 9, $P = 0\ 005$) Additionally, lower platelet counts (mean = 153,882/µl, $P < 0\ 0001$) were observed in the greyhounds as compared to non-greyhound controls Greyhounds also had lower total plasma proteins (mean 6 2 g/dl, $P = 0\ 0001$) than did non-greyhound controls. The lower hemoglobin P_{50} values in the greyhounds suggest that the increased concentrations of hemoglobin and increased PCV's previously reported in greyhounds are not due solely to selective breeding for superior racing abilities, but may also be the result of an unusual increased affinity of greyhound hemoglobin for oxygen These

data have practical implications for those involved in transfusion medicine, and for veterinarians treating the growing number of greyhound pets

Chapter 2

Introduction

Certain unique aspects of the greyhound hemogram are well known in the veterinary community Specifically, high PCV's, elevated hemoglobin concentrations, increased mean corpuscular volumes (MCV), and increased RBC counts have been reported in greyhounds as compared to non-greyhound dogs [1,2] Another study compared growing, racing and breeding dogs [3], but did not compare greyhounds to non-greyhound dogs, a fourth considered racing greyhounds of various ages [4] These studies utilized dogs from sources including kenneled research animals [1] and actively racing animals [3,4] Doxey's report [2] did not give demographic information on the study group The explanation for these elevated erythroid indices has traditionally been that intensive breeding for good racetrack performance has produced a selection pressure for increased total oxygen carrying capacity [3] There have been no similar reports regarding the platelet indices of greyhounds

With the increasing popularity of greyhounds as pets and the development of national "rescue" programs to place retired racing or breeding animals, veterinarians in small animal practice are more frequently seeing greyhounds as patients It is important to recognize laboratory peculiarities of certain breeds, lest physiologic variations from the species "normal" be interpreted as pathological. We utilized greyhounds from such a greyhound adoption program to characterize the hemograms, including platelet counts and platelet sizes, of the newly emerging retired greyhound pet Additionally, we evaluated parameters of hemoglobin

function -- hemoglobin P_{50} and 2,3 diphosphoglyceric acid (2,3 DPG) concentrations -- not normally used clinically to attempt to define the mechanism of increased hemoglobin concentrations and PCV's in the breed

Chapter 3

Materials and Methods

Study groups: Twenty-nine greyhounds obtained from two different breeding farms in Florida and Alabama were utilized Seven greyhounds already adopted as pets were also included The former animals were all recently transported from their origin to East Tennessee, and were awaiting adoption at the time of examination In all, 14 of 36 (39%) of the dogs were from farms in Florida, 17 (47%) were bred in Alabama, and 5 (14%) were from farms in the Northeastern United States Nine of 36 (25%) were sexually intact females, 5 (14%) were spayed females, 10 (28%) were intact males, and 12 (33%) were castrated males The mean age of the greyhounds was 4 3 years (range, 1 5-11 0 years)

The control animals were mixed breed dogs either housed in the laboratory animal facility at the university as teaching or research animals (n=14) or were pets volunteered by the owners for inclusion in the study (n=8) Those from the laboratory animal facility were either used as teaching animals in anesthesia laboratories, or were being conditioned between experimental protocols All laboratory control animals were obtained from USDA breeding facilities licensed in the State of Tennessee In the control group, 5 of 22 (23%) were sexually intact females, 4 (18%) were spayed females, 9 (41%) were intact males, and 4 (18%) were castrated males. Mean age of the control dogs was 3 4 years (range, 1 0-11 0 years)

Data Base All animals included in the study were given a full physical examination at the time of venipuncture A fecal flotation exam for ova was negative in all laboratory control dogs All client owned control dogs and greyhound dogs had had fecal flotation examinations in the previous year, and were maintained on prophylactic therapy for hookworm and ascarid infection (milbemycin or ivermectin) Blood samples from all farm greyhounds and laboratory control dogs, which had no history of prophylaxis for heartworm infection, were analyzed for microfilariae by a modified Knott's technique All client owned control dogs also had a Knott's test in this study, in addition, all had been tested for *Dirofilaria immitis* antigen in the previous year and were on monthly prophylaxis for heartworm infection (milbemycin or ivermectin)

Hematology: A single jugular venipuncture was performed on each animal, and the blood sample was immediately divided into evacuated containers containing EDTA, citrate, and heparin. PCV was evaluated using a standard microtechnique RBC and WBC counts were determined from the EDTA sample using a Coulter Counter (Coulter Counter Z_f, Coulter, Inc, Hialeah, FL) Differential white blood cell counts were performed manually using smears stained with a commercially available staining kit (Diff-Quick, Baxter/SP, McGaw Park, IL) Platelet counts were performed manually from the EDTA sample using phase microscopy Platelet size was determined using an Electrozone Celloscope (Particle Data Inc, Elmhurst, IL) coupled with a multichannel analyzer, as described by McDonald [5] Frequent calibration was performed using 2 02 µm diameter latex calibration beads

Concentrations of 2,3 diphosphoglyceric acid (DPG) (Sigma Chemical, St Louis, MO) and hemoglobin (Sigma) were determined by photometric methods using commercially

available kits Hemoglobin P_{50}'s were obtained using a Hemoscan oxygen dissociation analyzer (HEMOSCAN, Aminco, Silver Spring, MD) from fresh aliquots of blood diluted into EDTA In brief, a thin film of blood was placed under a gas permeable membrane, and the sample was placed in a humidified chamber. The sample was completely deoxygenated by purging the chamber with nitrogen, and then the sample was exposed to varying partial pressures of oxygen Percent saturation was measured using a spectrophotometric system with a tungsten-halogen light source Adjustments for variations in barometric pressure were made daily The dissociation reaction was carried out at 37°C, according to a standard protocol for analyzing human blood samples provided with the instrument Hemoglobin P_{50}'s were obtained from the dissociation curve by back-plotting the X-intercept from the 50% saturation point, and the Hill coefficient was calculated by measuring pO_2 at a second value for saturation, and using the method described by Stryer [6]

Total plasma protein concentrations were determined using a refractometer, and screening for microfilariae utilized the modified Knott's technique Reticulocytes were counted following staining with New Methylene Blue Serum samples from fourteen greyhounds, representing both farm and pet animals, were screened for antibodies to *Babesia canis* and *Ehrlichia canis* using immunofluorescent antibody methodology and commercially available antigen slides and control samples (Prota-Tek, St Paul, MN) Samples were screened at a concentration of 1 40 for *Ehrlichia*, and 1 20 for *Babesia*

Statistics: All data analysis was performed using the SAS system (SAS Institute, Cary, NC) The Student's t test was used to compare data from greyhounds with data from non-greyhound controls Linear correlation of platelet counts with PCV and P_{50}'s, and of

PCV's with P_{50}'s, was performed using the general linear models procedure (PROC GLM) in SAS

Chapter 4

Results

Several significant differences in hematologic parameters between the two groups of dogs were noted (Table I) As compared to non-greyhound controls, the greyhounds in this study were found to have higher concentrations of hemoglobin, higher PCV, lower total plasma protein, lower RBC numbers, higher MCV, lower platelet count, lower hemoglobin P_{50}, and lower Hill coefficient Typical oxygen-hemoglobin dissociation curves for greyhound and non-greyhound dogs are depicted in Figure 1 Reticulocyte counts were performed in fourteen of the greyhound dogs, with a mean value of 0 2% (range, 0 1-1 0%) No dog was found to be microfilaremic by Knott's test, and none of the fourteen tested greyhounds showed serologic evidence of exposure to *Ehrlichia* or *Babesia*

Significant linear correlations were found between PCV and platelet count (negative correlation, $P = 0\ 0006$, $r^2=0\ 19$), PCV and P_{50} (negative correlation, $P = 0\ 005$, $r^2=0\ 26$), and platelet counts and P_{50} (positive correlation, $P = 0\ 005$, $r^2=0\ 26$) The correlation between platelet counts and PCV is represented graphically in Figure 2

RBC count, PCV, P_{50}, and platelet count did not differ among different sexes (n=4) Also, there were no differences in any of the measured parameters between client owned (pet) control animals and control animals from the Laboratory Animal Facility The only differences between the pet greyhounds and those obtained from breeding farms was that the pet greyhounds had significantly lower WBC (mean 6235/ml vs 8436/ml, $P = 0\ 001$) and

segmented neutrophil (mean 3960/ml vs 6503/ml, $P = 0\ 002$) counts than did the greyhounds from farms This may be attributable to the higher incidence of abrasions in the farmed greyhound dogs, or to the poor sanitary conditions in the environment of the farmed dogs

Nineteen of 36 greyhounds (53%) were thrombocytopenic (range, 80,000 - 147,500/µl) by our laboratory reference range for canine platelet count (150,000 - 400,000/µl), whereas 1 of 22 (5%) of non-greyhounds was thrombocytopenic (platelet count = 132,500/µl)

Chapter 5

Discussion

Our results showing elevated red cell indices in greyhounds are generally in agreement with previous studies A notable exception is the evaluation of RBC number While other groups [1,2] have indicated increased RBC number in greyhounds, we report a significantly lower RBC count in this group of dogs We found no difference in MCH between the two groups, although our values for both groups were higher than traditional references ranges [7] This difference may be due to the different study populations among the reports However, our RBC counts were similar to those reported by Porter and Canaday [1] Heneghan [3] and Lassen *et al* [4] made no comparison between the RBC counts of greyhound and non-greyhound dogs

The issue of sample (patient) population is important in that conclusions drawn from a narrowly defined patient population may not be appropriate for the breed as a whole For example, our lack of serologic evidence of exposure to *Ehrlichia* or *Babesia* is in contrast to previous reports [8] This may be due to adequate tick control at the two farms from which the dogs were obtained Our selection criteria should produce conclusions applicable to greyhounds obtained through adoption programs for "retired" greyhounds or presented as privately owned pets for veterinary care

It has been the traditionally held view that the increased PCV and hemoglobin concentrations in greyhounds are results of intensive breeding selection for racing ability [3]

The results of this work imply that another mechanism, that of altered hemoglobin function, may also be operative The lower mean hemoglobin P_{50} in this group of greyhounds represents a "shift to the left" of the oxygen-hemoglobin dissociation curve, and reveals an underlying high affinity of greyhound hemoglobin for oxygen The lower mean Hill coefficient for the greyhound group suggests that a cooperative binding of oxygen to hemoglobin occurs to a lesser extent in greyhounds than in non-greyhounds Increased amounts of hemoglobin and increased PCV in these animals may be, in part, a compensatory change secondary to decreased oxygen delivery to the tissues The strong negative correlation between PCV and hemoglobin P_{50} in individual animals argues persuasively for a causal relationship There were no differences between greyhounds and non-greyhounds in the absolute plasma concentrations of 2,3 DPG, nor were there differences when the metabolite was expressed per unit hemoglobin or per unit PCV. This finding suggests that changes in 2,3 DPG concentrations were neither the cause of the altered hemoglobin function, nor have occurred in compensation for this altered hemoglobin function

The increased PCV in these dogs appears to be due to a macrocytosis, as RBC counts were lower than controls Our MCV values are consistent with an earlier report [4] This macrocytosis is not due to a reticulocytosis, and could represent a true physiologic macrocytosis analogous to the microcytosis of Akita dogs, [7] or the macrocytosis of poodles described by Schalm [9]

The decreased total plasma protein in the group seems even more significant in light of the lower plasma volumes in the breed (due to increased PCV) No mechanism for this

difference is evident from these data Differential albumin/globulin concentrations were not determined in these animals

We are unaware of previous reports of low platelet concentrations in normal greyhounds, although thrombocytopenia was documented in four greyhounds with a multi-cell line myeloproliferative disorder [10] One possible mechanism for this apparent physiologic thrombocytopenia is the stem-cell competition model of hematopoiesis [11] In this model, a bipotential stem cell (which is at a point in maturation distal to the multi-potential stem cell) may give rise to either committed red cell precursors or megakaryocytes A strong hormonal (i e erythropoietin or thrombopoietin) stimulus for the production of one cell line produces an increase in committed precursors for one cell line at the expense of the other In this case, a chronic mild hypoxia (predicted from a left shifted oxygen/hemoglobin dissociation profile, but not proved in this work) would result in EPO stimulation of the marrow and a predicted upregulation of erythropoiesis at the expense of megakaryopoiesis Considerable data have been amassed in support of this hypothesis, first using mice in hypoxia [12,13,14] and more recently in mice undergoing an erythropoietic stimulus from iatrogenic hyperthyroidism [15] In the current report, the strong correlation of platelet count with PCV and hemoglobin P_{50} suggests that the thrombocytopenia in these animals may be related to their altered red blood cell indices While the decreased RBC number reported herein seems counterintuitive, the possibility of altered circulating lifespan of RBC's in greyhounds should be considered, especially in light of the macrocytosis (since young RBC's are larger than old RBC's)

Other possible explanations for the finding of low platelet concentrations include splenic or pulmonary platelet sequestration, or a chronic low-grade immune-mediated process

resulting in decreased platelet life span Such a process has been suggested to occur secondary to numerous potential sources of antigenic stimuli [16]

There are several practical implications of this work Veterinarians treating greyhound pets should be aware that low platelet concentrations may be physiologic in the breed -- 51% of the animals in our group would be considered thrombocytopenic by non-greyhound reference ranges This may obviate the need for potentially time consuming and expensive workups of borderline low-platelet concentrations in greyhounds An alternate reference range for platelet counts in greyhounds suggested by these data would be 71,000 - 239,000/µl, although this is based on a relatively small number (n=36) of animals

Also, blood bankers and clinics using greyhounds as blood donors should be aware that greyhounds may not be the most appropriate donors for the preparation of platelet concentrates The possibility of an impaired oxygen delivering capacity in greyhound hemoglobin may also call into question the utility of greyhounds as red cell donors

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Appendix

Part 5

Figure 1

Typical oxygen-hemoglobin dissociation curves for greyhound (A) and non-greyhound (B) dogs The P_{50} values, calculated as indicated with the dotted line, were 25 0 and 30 0 mm O_2, respectively

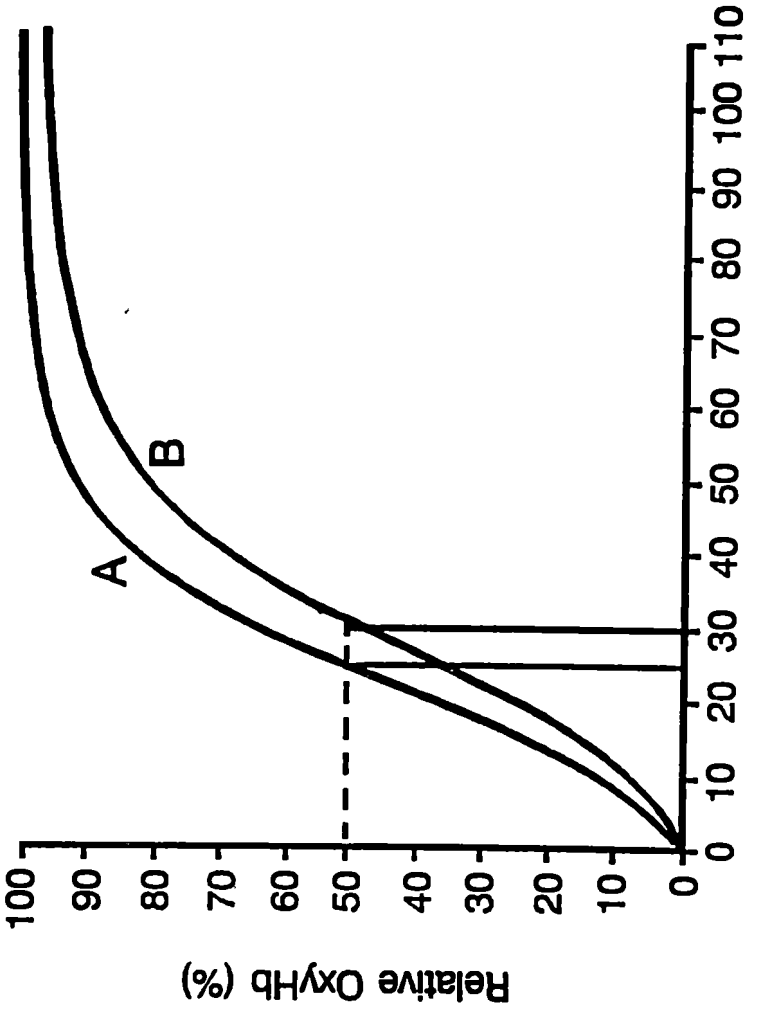
Relative OxyHb (%)
100
90
80
70
60
50
40
30
20
10
0
A
B
0 10 20 30 40 50 60 70 80 90 100 110
PO2 (mmHg)

Figure 1

Figure 2

The relationship between platelet count and PCV in a group of 58 greyhound (o) and non-greyhound (●) dogs PLT= platelet count x $10^3/\mu l$, and PCV = packed cell volume (%)

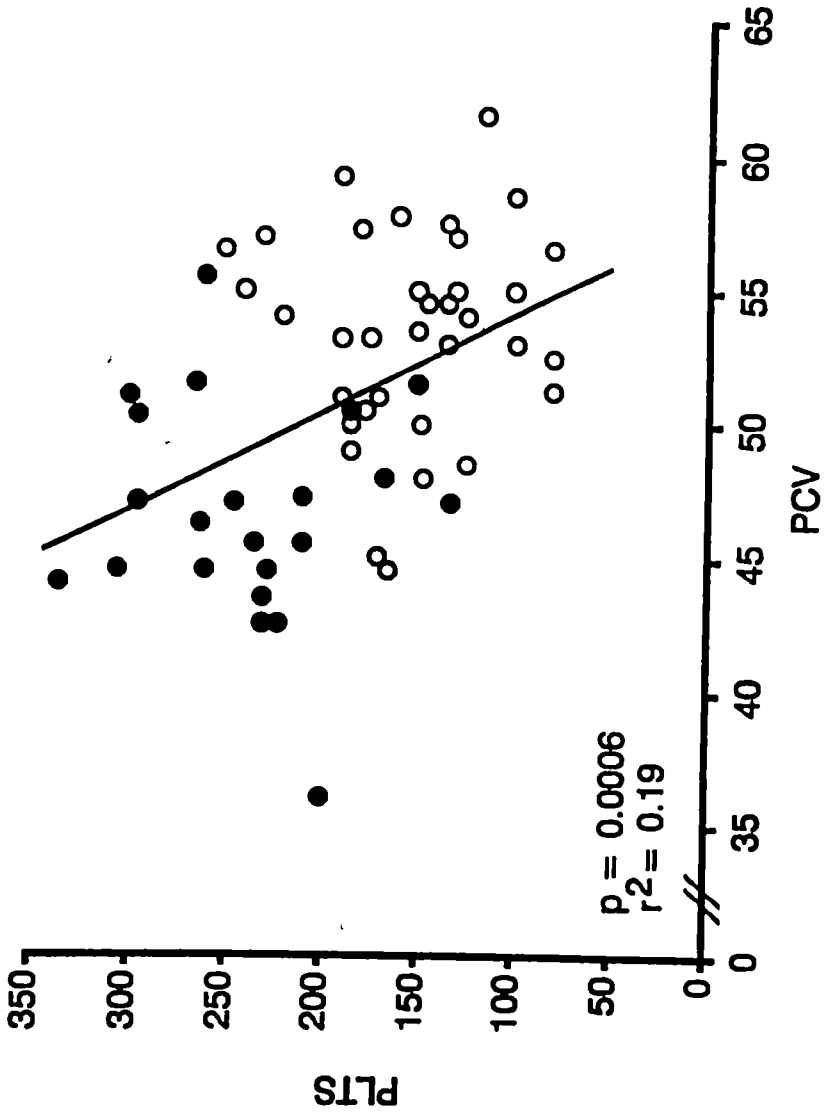
PLTS
350
300
250
200
150
100
50
0
0
35
40
45
50
55
60
65
PCV
p = 0.0006
r2 = 0.19

Figure 2

Table I

Hematologic parameters for greyhound (n=36) and non-greyhound (n=22) dogs Values are expressed as mean ± SD, with the number of individual observations indicated in parentheses *P* values are from the Student's t test

Table I

Parameter	Greyhound (n)	Non-Greyhound (n)	*P* value
Hemoglobin (g/dl)	19 86 ± 1 56 (36)	17 53 ± 1 31 (22)	<0 0001
PCV (%)	53 6 ± 3 8 (36)	46 6 ± 4 1 (22)	<0 0001
Total Protein (g/dl)	6 2 ± 0 4 (36)	6 7 ± 0 4 (22)	0 0001
RBC (x 10^{-6}/µl)	6 66 ± 0 67 (36)	7 10 ± 0 44 (22)	0 003
Mean Corpuscular Volume (µm^3)	81 0 ± 8 2 (36)	65 6 ± 2 9 (22)	0 0001
Mean Cellular Hemoglobin (pg/cell)	30 03 ± 3 09 (36)	24 68 ± 1 2 (22)	NS
Mean Cellular Hemoglobin Concentration (%)	37 10 ± 1 51 (36)	37 69 ± 2 00 (22)	NS
WBC (#/µl)	7886 ± 2560 (36)	7442 ± 2939 (22)	NS
Segmented Neutrophil (#/µl)	5867 ± 2285 (36)	4993 ± 2939 (22)	NS
Band Neutrophils (#/µl)	22 ± 51 (36)	3 ± 12 (22)	NS
Lymphocytes (#/µl)	1735 ± 839 (36)	2235 ± 1212 (22)	NS
Monocytes (#/µl)	194 ± 147 (36)	129 ± 142 (22)	NS
Eosinophils (#/µl)	74 ± 93 (36)	77 ± 143 (22)	NS
Basophils (#/µl)	5 ± 19 (36)	5 ± (22)	NS

Table I (cont.)

Parameter	Greyhound (n)	Non-Greyhound (n)	P-value
Platelet Count ($x\ 10^{-3}/\mu l$)	154 ± 43 (36)	238 ± 52 (22)	<0 0001
Platelet size (μm^3)	8 81 ± 1 46 (36)	8 37 ± 1 44 (22)	NS
2,3 DPG (mmol/ml)	2 34 ± 0 59 (19)	2 21 ± 0 26 (16)	NS
2,3 DPG/PCV (mmol/ml)	4 34 ± 0 94 (19)	4 74 ± 0 50 (16)	NS
2,3 DPG/Hb (mmol/g)	11 85 ± 2 52 (19)	12 78 ± 1 46 (16)	NS
Hemoglobin P_{50} (mm O_2)	23 5 ± 2 1 (11)	28.2 ± 1 5 (16)	< 0 0001
Hill Coefficient	1 9 ± 1 4 (11)	2.4 ± 0 4 (16)	0.005

Part 6

Effect of Thrombopoietin from Human Embryonic Kidney Cells on Erythropoiesis and Thrombocytopoiesis

Chapter 1

Abstract

Recent studies have shown that large doses of erythropoietin (EPO) administered daily over a 7-day period elevate erythropoiesis and lead to marked thrombocytopenia. Conversely, anemia was found in mice following stimulation of thrombocytopoiesis by an acute thrombocytopenic episode Although erythropoiesis and thrombocytopoiesis have been studied in mice after treatment with either hypoxia or EPO injection, only the effects of endogenous thrombopoietin (released after an acute episode of thrombocytopenia caused by an injection of anti-platelet serum) on erythropoiesis have been investigated Therefore, we injected mice with a potent source of thrombopoietin and evaluated both thrombocytopoiesis and erythropoiesis at 3 and 5 days after treatment The data show that thrombopoietin elevated thrombocytopoiesis with a concomitant reduction in erythropoiesis We found significantly elevated $\%^{35}S$ incorporation into platelets, platelet sizes, and total circulating platelet masses following thrombopoietin injections at both 3 and 5 days, hematocrits, reticulocyte counts, and total circulating red blood cell masses were reduced significantly in these same mice Compared to controls treated with human serum albumin, megakaryocyte size was increased on day 3, and megakaryocyte numbers were elevated on day 5 in mice treated with thrombopoietin Thrombopoietin did not change the blood volume of mice, but did cause an increase in splenic weight However, splenic sequestration of red blood cells was not the cause of anemia in mice treated with thrombopoietin, since splenectomized mice also showed increased thrombocytopoiesis with decreased erythropoiesis These data agree with previous studies showing an inverse relation between erythropoiesis and thrombocytopoiesis,

and are consistent with the hypothesis that the erythrocytic and megakaryocytic cell lines are in competition

Chapter 2

Introduction

The relationship between thrombocytopoiesis and erythropoiesis is complex, but it is clear that the two processes are not entirely independent *in vivo* Several reports in the mid-1970's [1-4] documented that stimulation of red blood cell production in mice following exposure to hypoxia results in a biphasic platelet response, with an initial increase in platelet counts and an eventual thrombocytopenia These data are consistent with either a direct effect of hypoxia on thrombocytopoiesis or with an erythropoietin (EPO) mediated mechanism Several studies documented that large doses of EPO given over a 2-day period of time (acute phase) would indeed elevate platelet counts of mice [5,6] McDonald et al. [7] administered large doses of human recombinant erythropoietin over a long period of time (chronic dose) to mice, and found a similar thrombocytopenia as was observed in mice held in continuous hypoxia In this study, after 7 days of EPO administration, platelet counts were reduced to 77% of control values, while packed cell volumes were increased to 128% of control values These data support the hypothesis of an EPO-mediated mechanism of suppression of platelet production, and not a direct marrow suppressive effect of hypoxia Therefore, these data provide an explanation for the finding of thrombocytopenia in mice exposed to hypoxia

Conversely, elevated thrombocytopoiesis induced by an acute thrombocytopenic episode was reported [7] to be associated with decreased red blood cell production in mice ($P <$

0 0005) five days after injection of rabbit anti-mouse platelet serum (RAMPS) These changes in the peripheral blood were inferred to be secondary to the action of endogenous thrombopoietin (a thrombocytopoiesis-stimulating factor, TSF) on the marrow In the present work, a partially purified preparation of TSF was injected into mice to test the hypothesis that the previously observed decreases in erythropoiesis observed following RAMPS administration occur via a TSF-mediated mechanism

Chapter 3

Materials And Methods

Animals Male C3H/HENHSD (C3H) mice (Harlan Sprague Dawley, Indianapolis, IN) 6 to 7 weeks of age were used in this work

Materials A partially purified Step II TSF obtained from human embryonic kidney (HEK) cell cultures was used in this study [8] The specific activity of the TSF was 3 93 units (U)/mg protein (2 55 mg protein/ml) The protein in this preparation was principally human serum albumin (HSA), which was added to the TSF preparation to stabilize the hormone A unit of TSF was previously defined as the amount of material (expressed in mg of protein) needed to increase $\%^{35}S$ incorporation into platelets of immunothrombocythemic mice to 50% above control levels [8] HSA, (Sigma Chemical Co , St Louis, MO) the carrier protein, was prepared to a final concentration of 2 55 mg/ml and was used as a negative control

No detectable levels of interleukin (IL)-1α, IL-1ß, or tumor necrosis factor were found in this TSF preparation [9] Furthermore, Step II TSF was tested for the presence of IL-6 and endotoxin Utilizing the ELISA assay, < 0 25 µg or < 2500 U of IL-6 (below the sensitivity of the assay) per U of TSF was present, also, < 2.5 U of endotoxin per U of TSF were detected by the E-toxate *Limulus* Amebocyte Lysate test (Sigma Chemical Company, St Louis, MO) We have previously provided evidence that > 40,000 U of IL-6 administered over a 2-day period [10] or > 50 U of endotoxin [9] were needed to stimulate platelet production in mice

Experimental Design Large doses of TSF were given to mice in order to stimulate thrombocytopoiesis On day 0, we administered subcutaneously (s c.) a total of 10 U/mouse of TSF, divided into 2 equal injections given eight hours apart All subsequent doses of TSF were given at the rate of 5 U/day divided twice daily (2.5 U/injection)

Mice that were killed on day 3 were injected with TSF s c 2 times each on days 0 and 1 (a total dose of 15 U of TSF/mouse) On day 2, each mouse was given intraperitoneally (i p) 30 μCi of $Na_2{}^{35}SO_4$ (ICN, Biomedicals Inc , Costa Mesa, CA), and on day 3 the animals were sacrificed

A second group of mice was given TSF s c twice daily on days 0-3 (total dose of 25 U of TSF/mouse), 30 μCi of ^{35}S were given i p on day 4, and the mice were sacrificed on day 5 Control mice were given the equivalent amounts of protein (HSA) on an identical injection schedule and treated with ^{35}S 24 hrs prior to assay

At the time of sacrifice, hematocrits, platelet counts, reticulocyte counts and RBC and WBC counts were determined on blood taken from the retroorbital sinus Five minutes after an i p. injection of a heparin-sodium pentobarbital solution [11], 0 5 ml of blood was collected via cardiac puncture into syringes containing 1 0 ml of 3 8% sodium citrate solution This blood was used for determination of both $\%^{35}S$ incorporation into platelets and platelet sizes Also, one femur was removed for measurement of megakaryocyte size and number, and the spleen was removed for determination of its weight Hematocrits were measured by a standard microtechnique and platelet counts were obtained manually by use of phase contrast microscopy Reticulocytes were counted in ten fields of 100 RBC's each from peripheral

blood smears that were stained with New Methylene Blue RBC and WBC counts were determined using a Coulter Counter Model Z_F (Coulter Electronics, Hialeah, FL).

Platelet Size Measurements: For platelet sizing, platelet-rich plasma (PRP) was obtained by centrifugation of whole blood at 160 g for 4 5 min at 22°C The tube containing the PRP was capped to minimize pH changes, which have been shown in previous studies to alter platelet sizes [12] Platelet sizing was performed using an Electrozone/Celloscope (Particle Data Inc , Elmhurst, IL) with a logarithmic scale as previously described [13] The instrument was set at log 10, current 6 and gain 4 Calibration was monitored frequently by using 2 02 μm diameter latex particles to standardize the instrument A 48 μm orifice was used for all size determinations

Percent ^{35}S incorporation into platelets: Percent ^{35}S incorporation into platelets was determined 24 hours after an i p injection of 30 μCi of ^{35}S in 0 5 ml saline The PRP not used for platelet sizing measurements was resuspended with the blood, and 1 ml of 1% EDTA in 0 538% NaCl was then added To obtain the PRP, blood was centrifuged at 450 g for 4 5 min at 4°C. PRP was then centrifuged at 800 g for 15 min at 4°C to obtain a platelet button. The platelets from each mouse were resuspended in 0 6 ml of 1% ammonium oxalate, mixed, centrifuged (15 min at 800 g), and washed one additional time with 0 5 ml of 1% ammonium oxalate A final wash in 1 5 ml of saline was made prior to counting platelet suspensions on the Celloscope and measuring radioactivity of the platelets. The percent ^{35}S incorporation into platelets was calculated as previously described [11]

Megakaryocyte Size and Number· To determine the size and number of megakaryocytes in the marrow of TSF and HSA-injected mice, the femurs that were removed

at time of sacrifice were fixed in 10% formalin, and histological sections were prepared as previously described [14] The sections were processed for light microscopy by embedding in glycol methacrylate, followed by staining with hematoxylin and eosin Megakaryocyte size was determined by randomly selecting sections of each femur and counting 200 megakaryocyte profiles using section profile perimeter measurements. Corrections for tissue shrinkage, section thickness, and optically lost profiles were made prior to statistical analysis of mean diameters and mean megakaryocyte numbers [14] The number of megakaryocytes was obtained from marrow sections at 400X magnification and is expressed as cells per mm^3 of marrow.

Blood Volume (BV) Other mice were injected with TSF or HSA as before, and BV in these animals was determined using ^{59}Fe-labeled RBC from donor mice [15] Six normal mice served as erythrocyte donors and were given a single i p injection of 0 5 μCi of ^{59}Fe, as ferric chloride (Dupont, Wilmington, DE) 48 hours before the mice were sacrificed At this time, blood was collected into sodium citrate and centrifuged 200 g for 15 min at 22°C The packed RBC were then mixed with saline to the volume of the original blood sample Both control (HSA injected) mice and TSF-injected mice were given 0 1 ml of ^{59}Fe-labeled RBC intravenously After 15 min, a sample of 100 μl of blood was taken from the retroorbital sinus, diluted into 2 ml water, and counted for radioactivity using a gamma scintillation spectrophotometer BV was expressed as milliliters of blood per 100 g body weight (%).

Total Circulating Platelet Mass (TCPM) and Total Circulating Red Blood Cell Mass (TCRBCM) TCPM was calculated by multiplying the platelet count for each mouse by the body weight (g) times the BV (%) times the average platelet size (μm^3) [15]

TCRBCM was calculated by multiplying the peripheral red cell count by the body weight (g) times the BV (%) times RBC mean cell volume (MCV) in cubic micrometers, which was calculated from RBC counts and hematocrits [16] These values were determined for each mouse to evaluate changes in the absolute platelet and RBC masses

Spleen Weight At the time of sacrifice, the spleen was removed and weighed Splenic weights are expressed as percent of total body weight for each mouse

Splenectomy Other mice were splenectomized 2 weeks prior to being placed in experiments Both normal and splenectomized mice were administered TSF or HSA using an identical protocol to earlier experiments, and platelet and RBC production were measured as described above

Statistics Statistical analysis of the data was performed using the PROC TTEST procedure in SAS

Chapter 4

Results

Figure 1 represents the combined results of three separate experiments in which mice were injected with TSF or HSA for 2 or 4 successive days, and platelet production indices were measured one day later Although platelets counts were unaltered (9 82 ± 0 30 x 10^5/µl for HSA-treated mice vs 8 61 ± 0 27 for TSF-treated mice on day 3, and 11 34 ± 0 42 x 10^5/µl vs 10 67 ± 0 34 on day 5), TSF caused significant ($P < 0\ 005$ to $P < 0\ 0005$) increases in 24-hour - %^{35}S incorporation into platelets and platelet sizes of mice, compared to values of HSA-treated controls on both days 3 and 5 In addition, significant decreases in hematocrits ($P < 0\ 05$ to $P < 0\ 0005$) and reticulocyte counts ($P < 0\ 05$ to $P < 0\ 0005$) were found in mice treated with TSF compared to HSA controls (Figure 2) Significant increases ($P < 0\ 0005$) in average spleen weight were found in TSF-treated mice compared to HSA-treated control mice, but BV values were not changed in TSF-treated mice compared to controls (Figure 3) Although not shown, WBC counts were not different between HSA and TSF treated mice on either day 3 (5,180 ± 1,800/µl vs 5,290 ± 1,490/µl, respectively) or on day 5 (5,180 ± 1,420/µl vs 5,820 ± 1,290/µl, respectively)

Figure 4 summarizes the results of TCPM and TCRBCM measurements of mice presented in Figure 1 In agreement with data presented above showing elevated thrombocytopoiesis in TSF-treated mice, Figure 4 also shows that TCPM was elevated ($P <$ 0 05) in mice given TSF at both 3 and 5 days compared to controls Conversely, a significant

decrease in TCRBCM was found on days 3 ($P < 0\ 05$) and 5 ($P < 0\ 0005$) in TSF-treated mice compared to controls

Figure 5 shows the results of megakaryocyte size and number determinations of mice presented in Figure 1 As shown, TSF elevated the size of megakaryocytes ($P < 0\ 025$) on day 3 and increased megakaryocyte numbers ($P < 0\ 0005$) on day 5 when compared to mice treated with HSA

Table I presents results of splenectomized mice which were given the same TSF- and HSA-treatment regimens as in the three previous experiments As before, platelet counts were unaltered, i e , $10\ 63 \pm 0\ 58 \times 10^5/\mu l$ for HSA-treated mice vs $9\ 52 \pm 0\ 55$ for TSF-treated mice on day 3, and $11\ 45 \pm 0\ 80 \times 10^5/\mu l$ vs $10\ 12 \pm 0\ 28$ on day 5 Significant increases in $\%^{35}S$ incorporation into platelets ($P < 0\ 0005$) and platelet sizes ($P < 0\ 001$) were found on day 3 The hematocrits were significantly reduced ($P < 0\ 001$) after TSF-treatment on day 5 in mice without spleens (Table I), along with significant decreases in the reticulocyte counts on days 3 ($P < 0\ 001$) and 5 ($P < 0\ 0005$) The reason for lack of significant differences in hematocrits between control and TSF-treated mice on day 3 and $\%^{35}S$ incorporation into platelets and platelet sizes on day 5 is probably because of the small numbers of mice used in this experiment. Regardless, the data show evidence of elevated thrombocytopoiesis and depressed erythropoiesis in splenectomized mice

Chapter 5

Discussion

The results of the present study show that large doses of TSF cause an increase in thrombocytopoiesis (elevated $\%^{35}S$ incorporation into platelets, larger platelets, and higher TCPM values) and megakaryocytopoiesis (increased size and number of femoral megakaryocytes) with a concomitant decrease in erythropoiesis (decreased hematocrits, reticulocyte counts, and TCRBCM), although BV was not altered In addition, there was evidence of increased platelet production in splenectomized mice with a concurrent decrease in red cell production These latter data support the conclusion that the changes in platelets and red cells were not due to increased BV or to splenic sequestration The finding of elevated thrombocytopoiesis with decreased RBC production agrees with the hypothesis of stem-cell competition [4,7] as the cause of anemia in TSF-treated mice As would be predicted by this model, a threshold dose of thrombopoietin exists; total doses of 1-2 U of TSF failed to induce anemia in prior experiments [8,10,12,13].

Several previous studies (recently reviewed [17]) reveal that chronic hypoxia causes erythrocythemia with marked thrombocytopenia The thrombocytopenia was not the result of dilution of the platelets in an expanded blood volume or of splenic sequestration, but was due to a decreased production of platelets as measured by reduced $\%^{35}S$ incorporation into platelets. Platelet life spans were unaltered in rats exposed to hypobaric hypoxia [3]. Decreased numbers of megakaryocyte precursor (small acetylcholinesterase positive,

SAChE+) cells [18], lower numbers of CFU-meg [19], and fewer mature megakaryocytes were found [20,21] in the bone marrow of mice exposed to hypoxia Hypoxic mice have elevated red blood cell masses [1-4] and decreased total circulating platelet counts [15] RBC transfusion into hypoxic mice alleviates the stimulus for increased erythropoiesis and, as expected, produces no changes in total circulating platelet counts [22,23] While all of these data are consistent with a competition between red cell and megakaryocyte precursors, platelet numbers may be reduced by other mechanisms, for example, Jackson et al [24] showed decreased platelet life spans in mice with high hematocrits resulting from hypertransfusion In support of this hypothesis is Jackson et al's [25] observation that chronic, severe anemia in animals results in thrombocytosis

While supraphysiologic doses of recombinant human EPO when observed in an acute phase seem to have non-specific stimulatory effects on thrombocytopoiesis [5,6], large chronic doses of EPO cause an increase in erythropoiesis and a decrease in thrombocytopoiesis [7] Also, megakaryocyte size in femoral marrow was unaltered by EPO injection, but the number of megakaryocytes was significantly reduced This work was confirmed recently when thrombocytopenia following EPO treatment was reported in baboons [26] Sullivan and McDonald [16] showed that another erythropoietic stimulus (thyroxine) resulted in increased erythropoiesis and decreased thrombocytopoiesis Hypothyroid dogs were also found to have increased platelet numbers associated with anemia [27] Fuchs and Eder [28] found a significant time effect on the differential leukocyte and platelet counts of rabbits after EPO treatment. They concluded that enhanced demand of one

cell line leads to a down-regulation of products from other cell lines All of these data are consistent with the hypothesis of stem-cell competition among cell lines in the marrow

In addition to the evidence showing that RBC and platelet production are inversely related, several biochemical similarities in erythroid and megakaryocyte precursors have been found For example, both erythrocytic and megakaryocytic cell lineages express common antigens (glycophorin A, glycoprotein III, and Plt-1), and share transcriptional factors [29-33]

The inverse relationship between platelet production and erythropoiesis suggested by these data is not observed under all conditions of stimulated erythropoiesis One reason is that investigators attempting to specifically stimulate production of one type of blood cell may not measure the other cell population in study animals Dose and schedule of injection may also have an effect on this inverse relationship, Yonemura et al [34] reported that administration of EPO to rats for 5 days resulted in a stimulation of megakaryocytopoiesis at all doses studied Berridge et al [5] administered EPO to rats and found an initial thrombocytosis (the first phase of the biphasic response seen in hypoxia), but no eventual thrombocytopenia. Clearly, there are situations in which simultaneous stimulation of red cell and platelet production occur [6] The reasons for these apparent discrepancies are unclear, but likely result from differences in doses, dosage schedules, and experimental time period. It appears that there are some conditions under which stimulation of the bipotential stem cell occurs, with no competition effects

We have recently reviewed examples of clinical conditions which are in agreement with the stem-cell competition hypothesis [17] However, thrombocytopenia is not routinely

reported in patients treated with exogenous EPO therapy, despite the widespread clinical use of this hormone This finding may be attributable to the doses or dosing schedules of EPO therapy, to eventual marrow compensation, or to a relatively insensitive endpoint (platelet count) In any case, it is important to realize that the changes in hematopoiesis produced by this proposed mechanism may not be of major significance clinically, and may not be recognized if they result in platelet counts or PCV's which, though lowered or elevated, do not fall outside of the reference range

While platelet counts in the present study were not increased in TSF-treated mice, more sensitive indicators of platelet production [13] showed increased thrombocytopoiesis Indices such as $\%^{35}S$ incorporation into platelets and platelet size measurements provide evidence of smaller and earlier increases in platelet production than do measurements of platelet count TCPM, which was increased in our TSF-treated mice, is an endpoint with physiologic significance, since Ebbe et al [35] reported that TCPM is more important than platelet count in the feedback system regulating thrombocytopoiesis

Because splenomegaly was observed in mice following administration of TSF, a small number of mice were splenectomized and treated with an identical regimen of TSF to test the hypothesis that splenic sequestration of RBC could be the mechanism for the observed decrease in RBC numbers in the peripheral blood In these splenectomized mice, elevated thrombocytopoiesis and decreased erythropoiesis were observed (Table I) However, these data also raise a question of the role of the spleen in the stem-cell competition hypothesis Although hematopoietic contributions of the spleen are likely to be minor in comparison to marrow [36,37], splenic hematopoietic cells could serve as a source of precursor cells which

might not possess pluripotentiality or might have a different differentiation potential. For example, Ebbe et al [38] found that transplantation of spleen cells into lethally irradiated recipient mice resulted in more megakaryocytes than did transplantation of the same number of marrow cells, suggesting that splenic stem cells have a different differentiation capacity than those of marrow This hypothesis could explain the larger and more persistent decreases in reticulocyte counts in splenectomized versus non-splenectomized TSF-treated mice

Work was presented in a previous study [7] showing that mice made acutely thrombocytopenic by administration of RAMPS had increased thrombocytopoiesis and decreased erythropoiesis The present data show that an exogenous source of TSF will also increase megakaryocytopoiesis and thrombocytopoiesis and decrease erythropoiesis. We report an increase in megakaryocyte size and number, and an elevation in platelet production (as measured by $\%^{35}S$ incorporation into platelets, TCPM, and platelet sizes) in mice treated with TSF. In the same mice, decreased erythropoiesis was found, as evidenced by reduced reticulocyte counts, hematocrits, and TCRBCM The anemia was not caused by increased blood volume or by splenic sequestration of RBC Therefore, these data support the hypothesis that the megakaryocytic and erythrocytic cell lines are in competition.

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Appendix

Part 6

Figure 1

Percent ^{35}S incorporation into platelets and platelet sizes of mice treated with a thrombocytopoiesis-stimulating factor (TSF) or, human serum albumin (HSA), the carrier protein Mice sacrificed on day 3 received a total of 15 U TSF and mice sacrificed on day 5 received a total of 25 U TSF each HSA was given at a protein concentration equal to that of TSF, i e , 8 37 mg HSA for mice sacrificed on day 3 and 13 95 mg HSA for mice sacrificed on day 5 The numbers on the bars represent the number of mice/treatment and the vertical lines represent the S E. Values for TSF-treated mice were significantly higher than values for HSA-treated mice $^{**}P < 0\ 005$, $^{***}P < 0\ 0005$

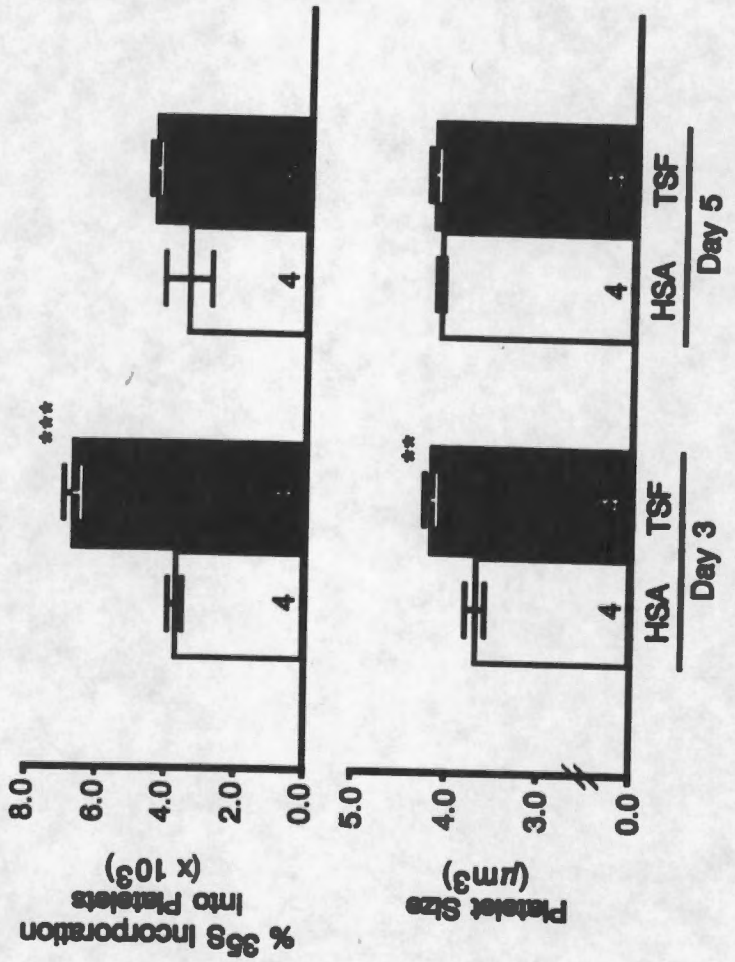
% 35S Incorporation into Platelets (x 10³)
8.0
6.0
4.0
2.0
0.0
***
4
4
Platelet Size (μm³)
5.0
4.0
3.0
0.0
**
4
4
HSA
TSF
Day 3
HSA
TSF
Day 5

Figure 1

Figure 2

Hematocrits and reticulocyte counts of the same mice presented in Figure 1 Values for TSF-treated mice were significantly lower than values for HSA-treated mice $*P < 0\ 05$, $***P < 0\ 0005$

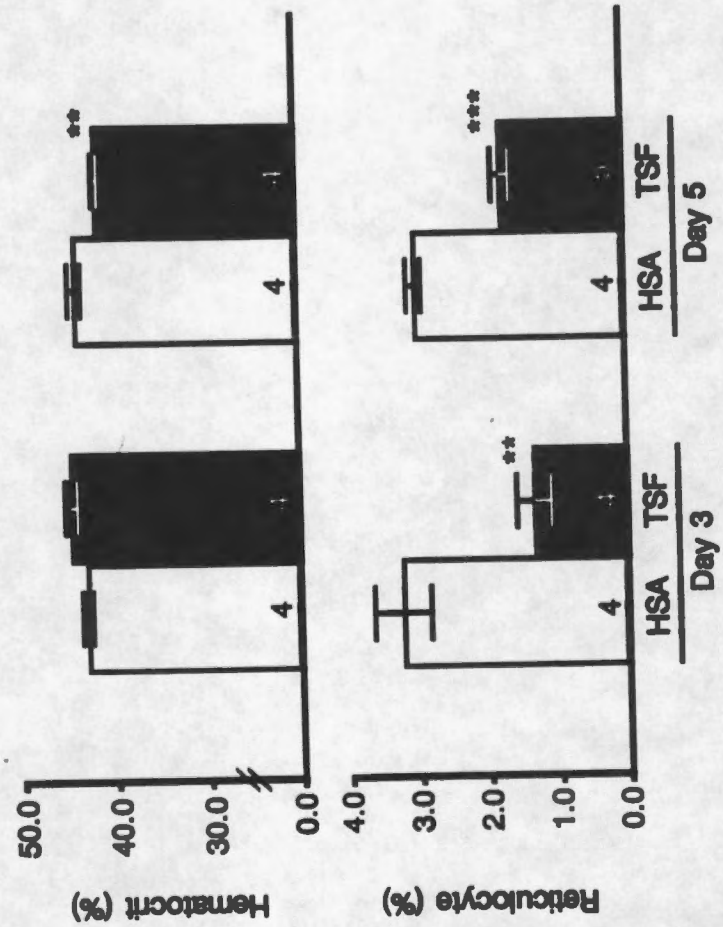
Hematocrit (%)
50.0
40.0
30.0
0.0
4
**
Reticulocyte (%)
4.0
3.0
2.0
1.0
0.0
**
***
HSA TSF
Day 3
HSA TSF
Day 5

Figure 2

Figure 3

Spleen weights and blood volumes of mice presented in Figure 1 The numbers on the bars represent the number of mice in each treatment group and the vertical lines indicate the S E. Values for TSF-treated mice were significantly greater than values for HSA-treated mice

$***P < 0\ 0005$

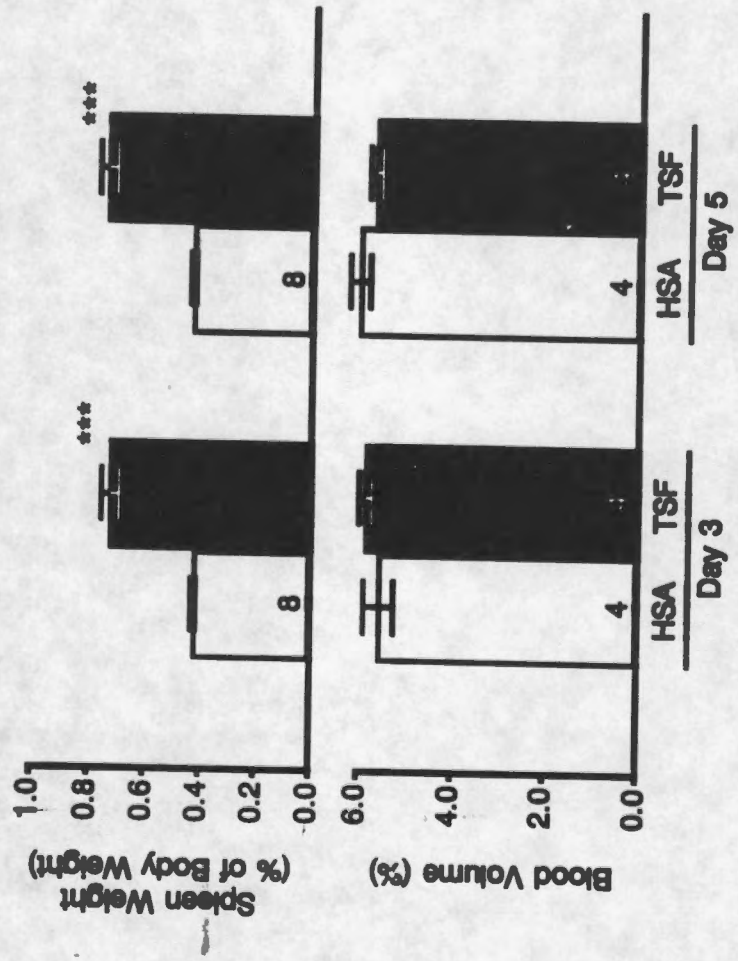
Spleen Weight
(% of Body Weight)
1.0
0.8
0.6
0.4
0.2
0.0
***
***
8
8
Blood Volume (%)
6.0
4.0
2.0
0.0
4
4
HSA
TSF
Day 3
HSA
TSF
Day 5

Figure 3

Figure 4

Total circulating platelet masses (TCPM) and total circulating red cell masses (TCRBCM) of mice presented in Figure 1 The numbers on the bars represent the numbers of mice for each treatment group and the vertical lines indicate the S E Values for TSF-treated mice were significantly different from values of HSA-treated mice $*P < 0\ 05$, $***P < 0\ 0005$

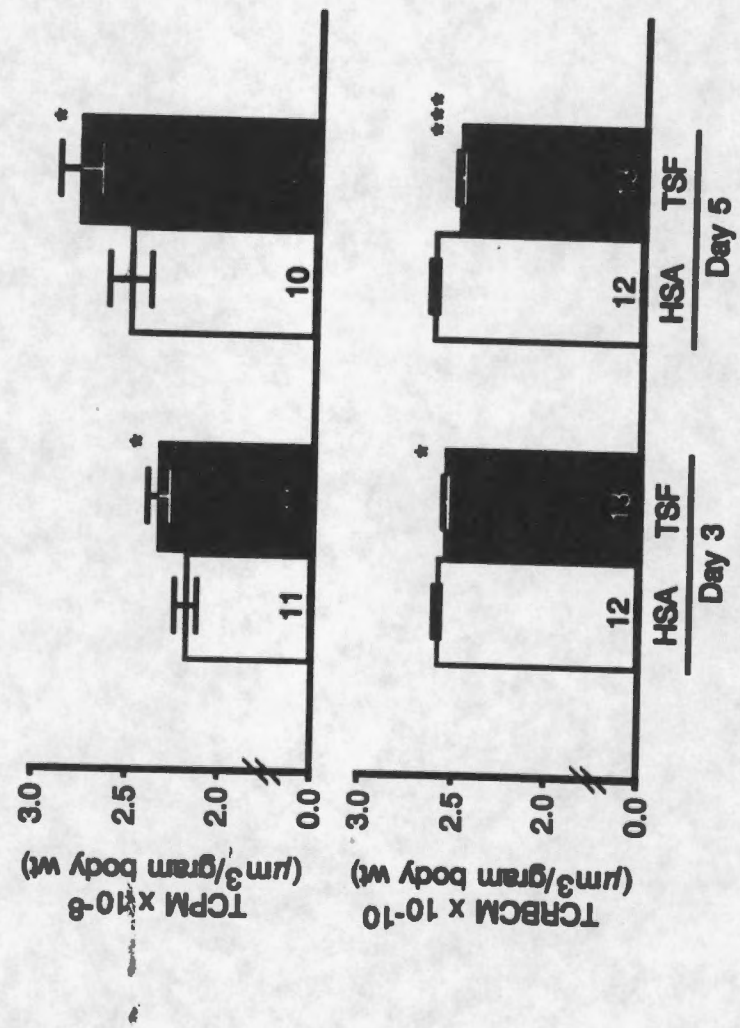
TCPM x 10-8
(μm3/gram body wt)
3.0
2.5
2.0
0.0
11
10
TCRBCM x 10-10
(μm3/gram body wt)
12
13
12
HSA TSF
Day 3
HSA TSF
Day 5

Figure 4

Figure 5

Average megakaryocyte diameter and number of mice presented in Figure 1 Five mice were utilized in each treatment group and the vertical lines represent the S E Values for TSF-treated mice were significantly greater than values for HSA-treated mice $*P < 0\ 025$, $***P < 0\ 0005$

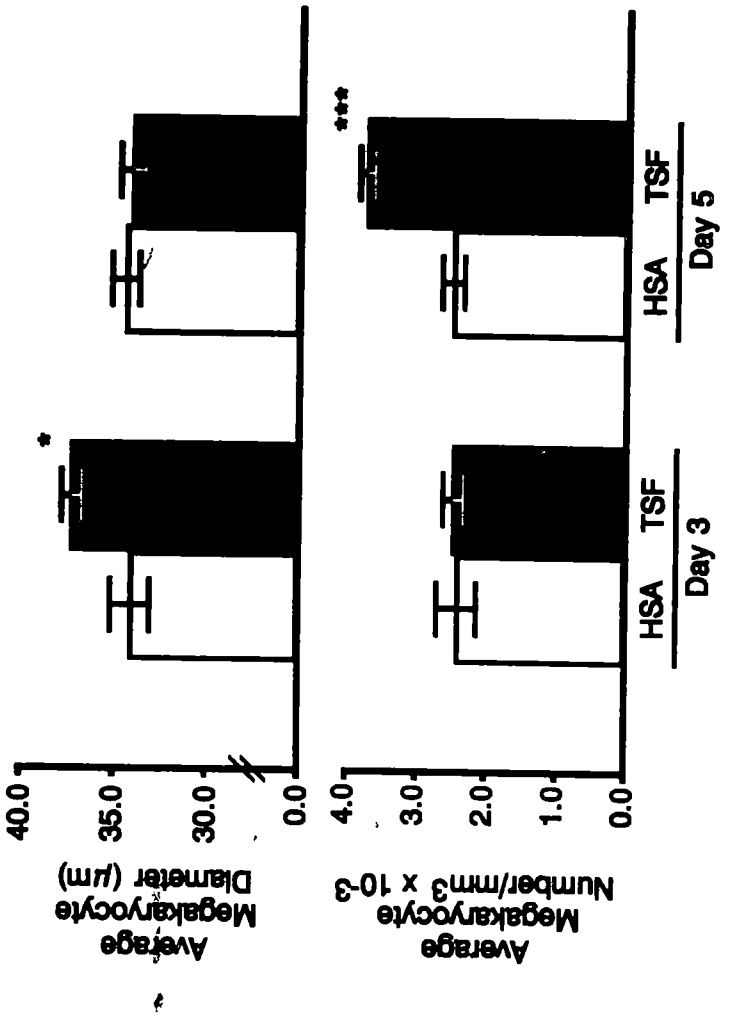
Average Megakaryocyte Diameter (μm)
40.0
35.0
30.0
0.0
*
Average Megakaryocyte Number/mm3 x 10-3
4.0
3.0
2.0
1.0
0.0
***
HSA
TSF
Day 3
HSA
TSF
Day 5

Figure 5

Table I

Effects of TSF on platelet and red blood cell production in splenectomized mice Values are presented as means ± S E , 4-5 mice were used for each treatment group Mice were splenectomized 2 weeks prior to treatment Injection schedule and doses of TSF and HSA are the same as indicated in footnote to Figure 1

Table I

	%^{35}S Incorporation Into Platelets (x 10^3)	Platelet Size (μm^3)	Hematocrit (%)	Reticulocytes (%)
Day 3.				
HSA	3 70 ± 0 20	3 67 ± 0 10	42 8 ± 0 5	3 22 ± 0 41
TSF	6 68 ± 0 25	4 16 ± 0 06	44 4 ± 0 7	1 32 ± 0 25
P	< 0 0005	< 0 001	NS	< 0 001
Day 5:				
HSA	3 43 ± 0 66	4 08 ± 0 04	43 8 ± 0 7	2 99 ± 0 10
TSF	4 41 ± 0 13	4 16 ± 0 05	41 4 ± 0 2	1 73 ± 0 13
P	NS	NS	< 0 001	< 0 0005

Part 7

Conclusions

The search for the pluripotent hematopoietic stem cell is one of the most important ongoing projects in the field of hematopoiesis Because early marrow progenitor cells are not identifiable based upon their morphology, attention has focused on antigenic markers which may allow identification of pluripotent populations of stem cells, this endeavor has practical implications, as physicians treating patients in whom marrow ablation is necessary therapeutically would like to be able to selectively repopulate the marrow with such cells One promising marker for hematopoietic stem cells is the CD34 antigen [1], which is a heavily glycosylated transmembrane protein found on about 1-2% of marrow cells Even this marker, however, is not specific for marrow pluripotent stem cells, as it also is expressed on precursors for B and T lymphocytes, and osteoclasts Much research in this field is directed towards the practical search for efficient marrow repopulating cell fractions, and is based upon flow cytometry and cell sorting of marrow using various surface markers

Another field which is producing substantial amounts of data is that of *in vitro* analysis of marrow cell cultures Marrow cultures are treated with various combinations of cytokines, and the resulting proliferation of cell lines is examined based upon morphology. Such methodology allows screening of high numbers of treatment combinations, but the information derived must always be interpreted with the caveat that *in vitro* data may not represent what occurs *in vivo* For example, interleukin-6 (IL-6) has shown promise in *in vitro* systems as a thrombopoietic agent, but its use in clinical medicine is limited by the often severe manifestations of inflammatory mediators These *in vitro* techniques should be utilized for their capacity for screening large numbers of treatment combinations, but caution should be exercised in extrapolation of data to living systems

Thus, the use of *in vivo* models is necessary for the study of hematopoiesis. As illustrated in this work, both designed experiments performed on terminal subjects and observational data are of value in hypothesis testing in the field of hematopoiesis The former allows the application of extreme stimuli and has the benefit of offering homogenous study populations (such as inbred strains of mice) which have less individual to individual variation, making observations of more marginal effects possible Clinical patients, on the other hand, allow insight into what the clinical significance of our laboratory observations may be

In the experiments with thyroxine detailed in this dissertation, thyroxine was found to cause increased erythropoiesis and a concurrent suppression of platelet production These effects were dose-dependent and time-dependent, with a maximal suppression of platelet counts occurring following 8 days of thyroxine treatment (platelet counts 50% of pretreatment values) The effect of the L-isomer was consistently greater than that of the D-isomer, suggesting that the two mechanisms by which thyroxine stimulates erythropoiesis are both active in producing the reciprocal decrease in thrombocytopoiesis As well, the detrimental effect on platelet production exerted by the L-isomer was significantly alleviated by the concurrent administration of propranolol (a non-specific β blocker), this also lends credence to the hypothesis of a direct relationship between thyroxine's effects on red blood cell and platelet production (i e both effects are partially reversed by a single antagonist).

Testosterone, another known stimulator of erythropoiesis, was also administered to mice in an attempt to delineate the relationship between red blood cell and platelet production Testosterone, like thyroxine, simulates EPO production, but unlike thyroxine, its direct effects are exerted at less mature stem cells (reportedly CFU-S) [2]. Thus,

administration of testosterone might be expected to have a mixed effect on thrombocytopoiesis since late regulators of erythropoiesis (like erythropoietin) have suppressive effects on platelet production, whereas a stimulation of immature precursor cells (i.e any cell maturationally proximal to the bipotential stem cell) should increase the production of both platelets and red cells. We found that the latter effect apparently predominates, as administration of testosterone resulted in significant increases in both red blood cells (PCV 104% of control on day 7, $P < 0\ 05$) and platelets (platelet count 110% of control on day 5, $P < 0\ 05$) Moreover, white blood cell counts did not change following castration or subsequent testosterone therapy, implying that testosterone produces a selective stimulation of the maturationally late bipotential colony forming cell proposed by Nicola and Johnson [3]

The clinical studies presented herein agree with the experimental data, although the desire to avoid invasive procedures in clinical patients restricts the clinical investigator to the observation of data from peripheral blood Given our findings that thyroxine had a detrimental effect on platelet production in mice, we were encouraged to examine the peripheral blood of veterinary patients with thyroidal disease The findings of altered platelet sizes in dogs with hypothyroidism and cats with hyperthyroidism, and of increased platelet counts in dogs with hypothyroidism, agree with findings in human beings with thyroid disease, and with the data from mice made iatrogenically hyperthyroid.

This latter benefit is equally evident in considering the findings of mild thrombocytopenia in greyhound dogs In fact, the idea for this project arose when several greyhound dogs were subjected to evaluation of platelet indices following an incidental

observation of thrombocytopenia, the awareness on the part of veterinarians of these apparent stem-cell competition effects will be of practical benefit in interpreting the hemograms of greyhound patients Additionally, the observation that 19% of the variation in PCV of all dogs is inversely correlated with variation in platelet counts gives an indication that competition effects may play a significant role in the maintenance of hematopoietic homeostasis in healthy animals.

Finally, we evaluated the converse side of the stem-cell competition hypothesis the effects of a strong thrombopoietic stimulus on red blood cell production As predicted by a competition model, thrombopoietin administration resulted in elevated indices of platelet production (i e $\%^{35}S$ incorporation into platelets) with a concomitant decrease in PCV The effects were dose dependent; positive linear correlations of thrombopoietin dose and megakaryocyte ploidy, $\%^{35}S$ incorporation into platelets, platelet size, and a negative correlation with PCV were observed

The ultimate resolution of the question of the relationship between red blood cell and platelet production may come in the form of the isolation of an antigenically distinct bipotential stem cell The demonstration of the behavior of such a cell may be demonstrated in *in vitro* culture systems In the meanwhile, observational studies of *in vivo* models of the stem-cell competition hypothesis have value in defining the physiology of hematopoiesis and the pathology of disease which affects the hematopoietic system For the astute observer, such studies may also have practical implications in medicine which cannot be anticipated at the inception of a study.

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General Appendix

Chapter 1

Background

A focus of this dissertation program was the evaluation of a novel method for the evaluation of megakaryocyte polyploidy using fluorescent *in situ* hybridization The conclusion of these efforts was that, because of the nature of the DNA probes available at this time, the technique was impractical in mice as an alternative to the evaluation of ploidy by flow cytometry It is the purpose of this appendix to review the methods of in situ hybridization employed, to document the DNA probes evaluated, and to document the evaluation of this technique

Megakaryocytes in the bone marrow undergo a process of endoreduplication which results in the production of large, polyploid cells in the later maturational stages of megakaryocytopoiesis Polyploidy has traditionally been assessed using flow cytometry, with detection of total nuclear fluorescence following staining with propidium iodide Based on this methodology, a great deal of information has been amassed describing the behavior of megakaryocyte ploidy in health, and with various experimental manipulations There is a relative paucity of data regarding changes in megakaryocyte ploidy in human beings with disease states, in part because the evaluation of megakaryocyte ploidy by flow cytometry requires the collection of a marrow aspirate into a liquid medium, clinicians routinely collect marrow aspirate samples and immediately express them onto glass slides for cytological evaluation. As well, it is difficult to obtain adequate samples for the evaluation of

megakaryocyte ploidy in pediatric patients, due to the small aspirate yields In response to these latter problems, we proposed to develop a technique for evaluating megakaryocyte ploidy which could be conducted from the routinely collected marrow aspirates, or performed retrospectively on aspirates

Megakaryocyte ploidy is reported in two fashions the modal ploidy class, and then geometric mean of ploidy state for all megakaryocytes observed. The former index is useful for describing the normal distribution of ploidy classes, which vary with species or strain; for example, most mice have a modal ploidy class of 16N, whereas the C3H strain of mice have a modal ploidy class of 32N There is also species variability in ploidy, with rabbits and monkeys having a modal ploidy class of 8N, and humans demonstrating modal 16N ploidy. Expressing ploidy as geometric mean of all megakaryocytes observed allows for a more quantitative and sensitive assessment of changes in ploidy, and while this number (which is a continuous variable) is not a physiologically intuitive index, it is useful in experimental medicine to describe the endoreduplicative behavior of megakaryocytes

In order to determine either modal ploidy class or geometric mean, the researcher needs a distribution of megakaryocytes in the various ploidy classes Our proposal was to evaluate the ploidy state of megakaryocytes on an individual basis by counting the copies of the Y chromosome, as visualized with a fluorescent DNA probe under fluorescence microscopy The Y chromosome was chosen as the initial ploidy index to minimize counting of signal, and to decrease clutter effects as the technique was being developed and evaluated. Based on these observations, the modal ploidy class could then be assigned from a histogram, and geometric mean ploidy could be calculated.

In this study, we attempted to develop and validate the technique of determination of megakaryocyte ploidy by *in situ* hybridization A highly purified preparation of thrombopoietin was injected into mice in varying doses, and red cell indicies and endpoints of platelet production were evaluated Megakaryocyte ploidy was to be evaluated by flow cytometry as a "gold standard", and by *in situ* hybridization In addition to validation of the new technique, this experimental deisgn also afforded the opportunity to document the dose-response characteristics of the response of megakaryocyte ploidy and PCV to thrombopoietin administration, these relationships have not been previously reported

Chapter 2

Materials and Methods

Animals Male C57BL (C57BL/6NHSD) mice (Harlan Sprague Dawley, Indianapolis, IN) 6 to 7 weeks of age were used in this work

Materials A partially purified Step II TSF obtained from human embryonic kidney (HEK) cell cultures was used in this study [1] The specific activity of the TSF was 45 5 units (U)/mg protein (0 17 mg protein/ml) The protein in this preparation was principally human serum albumin (HSA), which was added to the TSF preparation to stabilize the hormone A unit of TSF was previously defined as the amount of material (expressed in mg of protein) needed to increase $\%^{35}S$ incorporation into platelets of immunothrombocythemic mice to 50% above control levels [1] HSA, (Sigma Chemical Co , St Louis, MO) the carrier protein, was prepared to a final concentration of 0 17 mg/ml and was used as a negative control.

No detectable levels of Interleukin (IL)-1α, IL-1ß, or tumor necrosis factor were found in this TSF preparation [2] Furthermore, Step II TSF was tested for the presence of IL-6 and endotoxin Utilizing the ELISA assay, < 0 25 µg or < 2500 U of IL-6 (below the sensitivity of the assay) per U of TSF was present, also, < 2 5 U of endotoxin per U of TSF were detected by the E-toxate *Limulus* Amebocyte Lysate test (Sigma Chemical Company, St Louis, MO) We have previously provided evidence that > 40,000 U of IL-6 administered over a 2-day period [3] or > 50 U of endotoxin [2] were needed to stimulate platelet production in mice

Experimental Design: For dose response experiments, mice were administered total doses of 0 25 U to 4 0 U of thrombopoietin, divided into four equal subcutaneous injections and administered twice daily on days 0 and 1 Mice were administered 30 μCi of ^{35}S 24 hours prior to assay, and were sacrificed on day 3

At the time of sacrifice, hematocrits, platelet counts, and reticulocyte counts were determined on blood taken from the retroorbital sinus Five minutes after an i p injection of a heparin-sodium pentobarbital solution, 0 5 ml of blood was collected via cardiac puncture into syringes containing 1 0 ml of 3 8% sodium citrate solution This blood was used for determination of both %^{35}S incorporation into platelets and platelet sizes

Platelet Size Measurements: For platelet sizing, platelet-rich plasma (PRP) was obtained by centrifugation of whole blood at 160 x g for 4 5 min at 22°C The tube containing the PRP was capped to minimize pH changes, which have been shown in previous studies to alter platelet sizes [4] Platelet sizing was performed using an Electrozone/Celloscope (Particle Data Inc., Elmhurst, IL) with a logarithmic scale as previously described [5]. The instrument was set at log 10, current 6 and gain 4. Calibration was monitored frequently by using 2 02 μm diameter latex particles to standardize the instrument. A 48 μm orifice was used for all size determinations

Percent ^{35}S incorporation into platelets: Percent ^{35}S incorporation into platelets was determined 24 hours after an intraperitoneal injection of 30 μCi of ^{35}S in 0 5 ml saline. The PRP not used for platelet sizing measurements was resuspended with the blood, and 1 ml of 1% EDTA in 0 538% NaCl was then added To obtain the PRP, blood was centrifuged at 450 x g for 4 5 min at 4°C PRP was then centrifuged at 800 g for 15 min at 4°C to obtain

a platelet button The platelets from each mouse were resuspended in 0 6 ml of 1% ammonium oxalate, mixed, centrifuged (15 min at 800 x g), and washed one additional time with 0 5 ml of 1% ammonium oxalate A final wash in 1 5 ml of saline was made prior to counting platelet suspensions on the Celloscope and measuring radioactivity of the platelets The percent ^{35}S incorporation into platelets was calculated as previously described [6]

Megakaryocyte Ploidy Analysis: For measurement of megakaryocyte DNA content, a sample of marrow was collected by flushing a femur and two tibias with 1 0 ml of a solution of CATCH media The media contained citrate, adenosine and theophylline in Hanks medium [7] The media was free of calcium and magnesium, and was supplemented with BSA and DNAse The marrow suspensions collected into plastic tubes to prevent megakaryocyte adhesion to glass surfaces. The marrow suspensions were filtered through a nylon membrane with a 105µm pore size (Small Parts Inc, Miami, FL) The cells were then incubated with a saturating concentration (total 10 µl/ sample) of a monoclonal anti-mouse platelet antibody (4A5) [8] for 30 min at 4°C Each sample was then washed once with 3 0 ml/wash of CATCH medium Cell suspension were pelleted between washes by centrifugation for 5 mins at 160 x g in a 4°C centrifuge Each sample was then incubated with 10 µl of fluoresceinated goat anti-rat immunoglobulin $F(ab')_2$ fragments (TAGO, Burlingame, CA) for 30 min at 4°C Following an additional wash with CATCH media, the FITC labeled cells were then resuspended in 3 0 ml of a solution of propidium iodide (50 µg/ml). The marrow cells were analyzed on an EPICS 753-Flow Cytofluorometer (Coulter Electronics, Hialeah, FL) From 300-800 x 10^3 propidium iodide positive cells were examined per sample, of the propidum iodide positive cells analyzed, the DNA content of from 368 to 1028 4A5-positive cells was

analyzed. Total numbers of cells corresponsing to each ploidy class were calculated from the cytometer, and results are reported as the geometric mean of the data for each mouse

DNA probes: DNA probes were obtained from several sources The PY353 probe [8] was generously provided by Dr Colin Bishop, the 145SC5 probe [9] was provided by Dr Yukata Nishioka, the probes designated PY1, PY2, and PY3 [10] were supplied by Dr Ed Palmer, and the probes Sx1 1, Sx 1 3, Sx2 6, and Sx 2 7 [11] were provided by Dr. Michael Mitchell Probes were prepared for hybridization by either purification over a DNA purification resin (Qiagen, Inc Chatsworth CA), cesium chloride banding [12], or both

Labeling of probes: Purified DNA preparations were labeled with digoxigenin by nick translation Briefly, 1 μg of DNA was incubated at 15°C for 1 hour in the presence of 0 02 mM dATP, 0 02 mM dCTP, 0 02mM dGTP, 0.01 mM dTTP, 0 01 mM digoxigenin-dUTP (all from Boehringer Mannheim, Indianapolis, IN), 50mM Tris (pH 7 8), and 1X nick translation enzyme mixture (BioNick, BRL/Gibco, Grand Island, NY) The reaction mixture was then passed over a nick translation Sephadex G-50 column (Pharmacia LKB Biotechnology, Piscataway, NJ), and the resulting small (< 500 bp) fragments collected The digoxigenin incorporation into the probe and recovery from the column were monitored by radiolabelling with ^{3}H-TTP (ICN pharmaceuticals Costa Mesa, CA)

Preparation of cell suspensions: Femoral and tibial marrow were collected from sacrificed mice by flushing the bones with a solution of CATCH medium, containing citrate, adenosine and theophylline in Hanks medium [7] The marrow cells were pelleted by centrifugation at 800 x g for 5 minutes at 22°C, and were resuspended in 8-10 ml of 0.8% sodium citrate Following incubation for 20 minutes at 22°C, 0 5 ml of freshly prepared

Carnoy's fixative (75% methanol, 25% glacial acetic acid) was added to the samples Following an identical centrifugation, cell pellets were resuspended in 2-3 ml of pure Carnoy's fixative, with immediate subsequent centrifugation The cell pellets were then washed two additional times with Carnoy's fixative, with 20 minute incubations at 22°C between washings Cells thus fixed were stored at 0°C in 1 ml of Carnoy's solution until the time of analysis

At the time of analysis, cells were warmed to 22°C, gently resuspended and applied to standard glass microscope slides at 4°C These slides were air dried for at least 48 hours prior to hybridization

***In situ* hybridization conditions:** Probes were prepared by drying 50 ng of purified DNA (and competitor DNA (cot DNA, Gibco BRL, Grand Island, NY), if required) in a warmed, evacuated centrifuge for approximately 30 minutes [13] The DNA was then resuspended in a hybridization solution consisting of 50% formamide, 2X SSC (i e 0 3 M NaCl, 0 025 M NaH_2PO_4,), and 10% dextran While the solution of probe DNA was denatured by incubation at 70°C for 5 minutes, marrow slides were denatured for 30 seconds in 70% formamide/2X SSC, and were subsequently dehydrated in an ethanol series (70%, 80% and 95%, for 1 minute each) The probe solution was then applied to the dehydrated cellular sample, sealed with rubber cement and placed in a humidified incubation chamber at 37°C overnight (12-16 hours)

Detection protocol: The following day, the hybridized slides were washed for 5 minutes in a solution of 2X SSC at 37°C, with gentle manual agitation The slides were then stored in a solution of PN (0 1 M Na_2HPO_4, 0 009M NaH_2PO_4, 0 11% Nonidet P-40 (Sigma, St

Louis, MO)) while detection reagents were prepared Detection reagents were 1.5% fluoresceinated anti-digoxigenin of sheep origin ("anti-dig", Boeringer-Mannheim, Indianapolis, IN) and 1 0% fluoresceinated goat anti-sheep IgG ("anti-sheep IgG", Boeringer-Mannheim, Indianapolis, IN) in 4X SSC and 1% bovine serum albumin (BSA, Sigma, St Louis, MO) After removal from the PN soaking solution, each slide was covered with 200 µl of blocking solution (4X SSC in 1% BSA), with a subsequent 5 minutes incubation in the dark at 22°C A total of 100µl of anti-dig detection reagent per slide was then applied. The slides were incubated in the dark, in a humidified chamber at 22°C for 45 minutes Following 2 washes in PN solution (2 minutes each with gentle agitation), the slides were again covered with 100 µl of blocking solution and incubated for 5 minutes 100 µl of the anti-sheep IgG detection reagent was then applied per slide, with a subsequent 45 minute incubation The slides were washed an additional 2 times, 2 minutes per wash in PN Finally, 25 µl of propidium iodide counterstain solution (2% diazobicyclo-octane (Sigma, St Louis, MO), propidium iodide 0 5 µg/ml, 0 02 M Tris, 90% glycerol) was applied The slides were allowed to sit at 4°C for 5 minutes before viewing The specimens were then examined under a fluorescence microscope at 1000 X, using a broad band fluorescence filter

Statistics: The PROC GLM procedure in SAS was used to prepare an analysis of variance and construct linear regression models of the data

Chapter 3

Results

Significant positive correlations were demonstrated between dose of thrombopoietin and platelet size, $\%^{35}S$ incorporation into platelets, megakaryocyte ploidy, and packed cell volume These relationsips are illustrated in Figures 1-4 Of particular note are the linear, inverse relationships between packed cell volume and thrombopoietin dose (negative correlation) and platelet indices and thrombopoietin dose (positive correlation) The megakaryocyte ploidy data reported were obtained only by flow cytometry Significant efforts were made to obtain parallel data by *in situ* hybridization, but this technique was determined to be impractical for analysis of megakaryocyte ploidy.

The PY353 and 145SC5 probes produced a diffuse pattern of fluorescence along the Y chromosome which allowed visualization of the chromosome in ruptured nuclei. Because these probes produced a diffuse "painting" effect, it was not possible to enumerate copy number in interphase nuclei of polyploid cells Attempts to further amplify the signal intensity by subsequent layering of detection reagents resulted in excessive background fluorescence which precluded interpretation

Probes PY1, PY2, and PY3 produced excessive (non-Y chromosome) fluorescence in the absence of competitor DNA, and therefore required competition with cot DNA Total observed fluorescence decreased significantly following competition, with weak residual fluorescence on exteriorized chromosomal material remaining This signal was inadequate

for detection of copy number in polyploid nuclei, in which counterstain intensity was high

Probes Sx1 1, Sx1 3, Sx2 6, and Sx2 7 also required competition for ubiquitous sequences, with a similar competitor dose-dependent decrease in residual fluorescence As with the PY series probes, nuclear depth and counterstaining intensity precluded accurate enumeration of copy number in polyploid nuclei Attempts to increase signal intensity, by alternating application of anti-dig and anti-sheep IgG reagents, resulted in increasing background intensity in a dose-dependent fashion

Chapter 4

Discussion

The dose-response characteristics of indices of platelet production (platelet size, $\%^{35}S$ incorporation into platelets, and megakaryocyte ploidy) and red blood cell production (PCV) were consistent with the stem-cell competition hypothesis already reviewed [14] (i e , inverse relationship) These data are the first description of linear dose-response characterstics of megakaryocyte ploidy and PCV in response to thrombopoietin administration The data on megakaryocyte ploidy were obtained only by flow cytometry, as significant technical impediments prevented the collection of data by *in situ* hybridization

The central problem encountered in the application of fluorescent *in situ* hybridization to the enumeration of megakaryocyte polyploidy in bone marrow resides in the absolute need for focal, intense fluorescent signal in hybridized nuclei The probes evaluated in this work were inadequate for this purpose because they either produced a more diffuse, "painting" type signal (PY353 and 145SC5), or because they produced a focal fluorescent signal with inadequate intensity to allow enumeration through the thick nuclear composition on polyploid cells and the couterstaining fluorescence Thus, we conclude that the probes evaluated in this work are not appropriate to make the technique of enumeration of megakaryocyte ploidy by *in situ* hybridization practical in mice

There are a number of factors which determine what the fluorescence pattern and intensity of a particular probe will be These are primarily probe size, target size, target

and target composition Probe size is determined by the methodology of obtaining the probe DNA, and varies from cosmid sized probes, to larger cosmid vectors, to the even larger YAC DNA carriers In this work, probes from several hundred (PY353, 145SC5) to several thousand (Sx series probes) base pairs were evaluated from plasmid and cosmid sources No larger segments of YAC DNA were evaluated The probe size interacts critically with the target size, i e the number of target sequences present in the target genome The smallest probes evaluated in this work had an acceptable target product, since they detected sequences with large copy numbers For example, the 145SC5 probe was a small (plasmid) probe size, but had a genomic target of approximately 200 copies These factors, in turn, depend on the distribution of the copy *distribution*, in this case, the 200 copies were widely distributed and thus produced a diffuse pattern of fluorescence The Sx series probes were larger, but contained DNA from coding sequences with much lower copy numbers Finally, target composition bears on fluorescent intensity If the target sequences have significant regions of repeated DNA elements which also appear on other mouse chromosomes, these sequences must be "competed" out using a source of DNA enriched in repeat elements (such as cot DNA) These competed sequences are non-hybridized and are removed during washing, and thus do not contribute to the final fluorescent intensity.

The probes evaluated have all been applied successfully to the sexing of mouse embryos or tissues, but the different demands of sexing by autoradiography and enumeration of copy number by *in situ* hybridization must be recognized In autoradiography, the distribution of the copies of the target DNA is less important, since with adequate exposure time a confluent area of exposure is created As well, compensation for ubiquitous sequences can be

controlled by a combination of competition and varying exposure time Finally, the nuclear depth is an impediment to enumeration of copy number, as focusing through the couterstained nuclei is necessary to detect all copies of the target sequence As well, the large amount of nuclear material in polyploid cells produces a significant intensity of counterstain fluorescence, which further complicates enumeration This latter problem was somewhat alleviated in these experiments by decreasing or eliminating counterstain concentrations, although this manipulation made distinguishing nuclear boundaries difficult

Thus, the technique of polyploidy determination in marrow specimens by *in situ* hybridization is impractical in mice given the currently available sources of probe DNA However, the concept still holds promise in the evaluation of marrow specimens from human beings, since appropriate probes to various chromosomes in the human genome are commercially available. Future experiments are planned in which such probes will be applied to the marrow of HIV positive human beings with and without the thrombocytopenia of HIV/AIDS, in an attempt to determine the behavior of megakaryocyte ploidy in HIV related thrombocytopenia

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Appendix

General Appendix

Figure 1

Linear correlation of dose of thrombopoietin and platelet size in mice Points indicate mean platelet size of all mice receiving that dose of thrombopoietin, the vertical bars indicate standard error The total number of mice in the regression analysis was 22

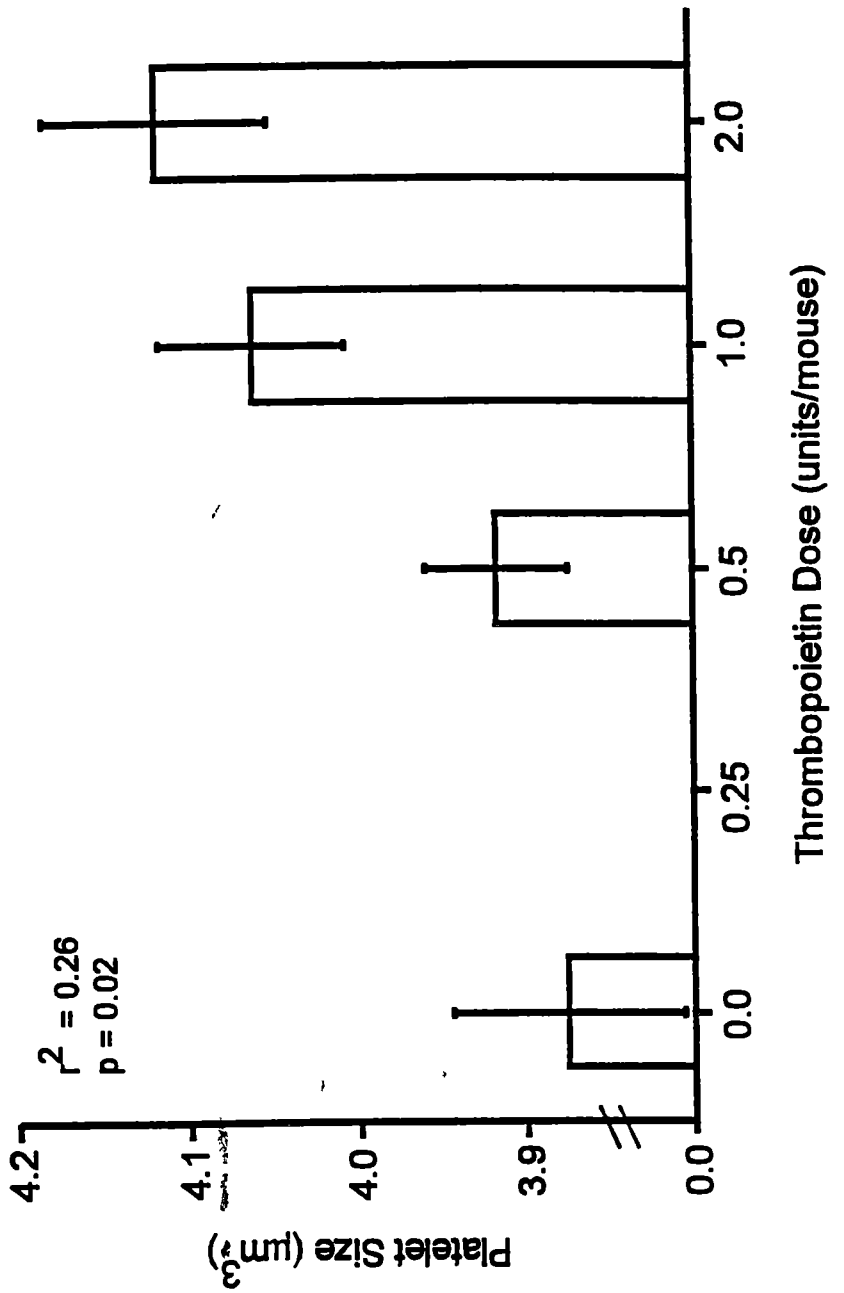
Platelet Size (μm³)
4.2
4.1
4.0
3.9
0.0
r² = 0.26
p = 0.02
0.0
0.25
0.5
1.0
2.0
Thrombopoietin Dose (units/mouse)

Figure 1

Figure 2

Linear correlation of dose of thrombopoietin and % ^{35}S incorporation into platelets in mice Points indicate mean % ^{35}S incorporation into platelets of all mice receiving that dose of thrombopoietin, the vertical bars indicate standard error The total number of mice in the regression analysis was 57

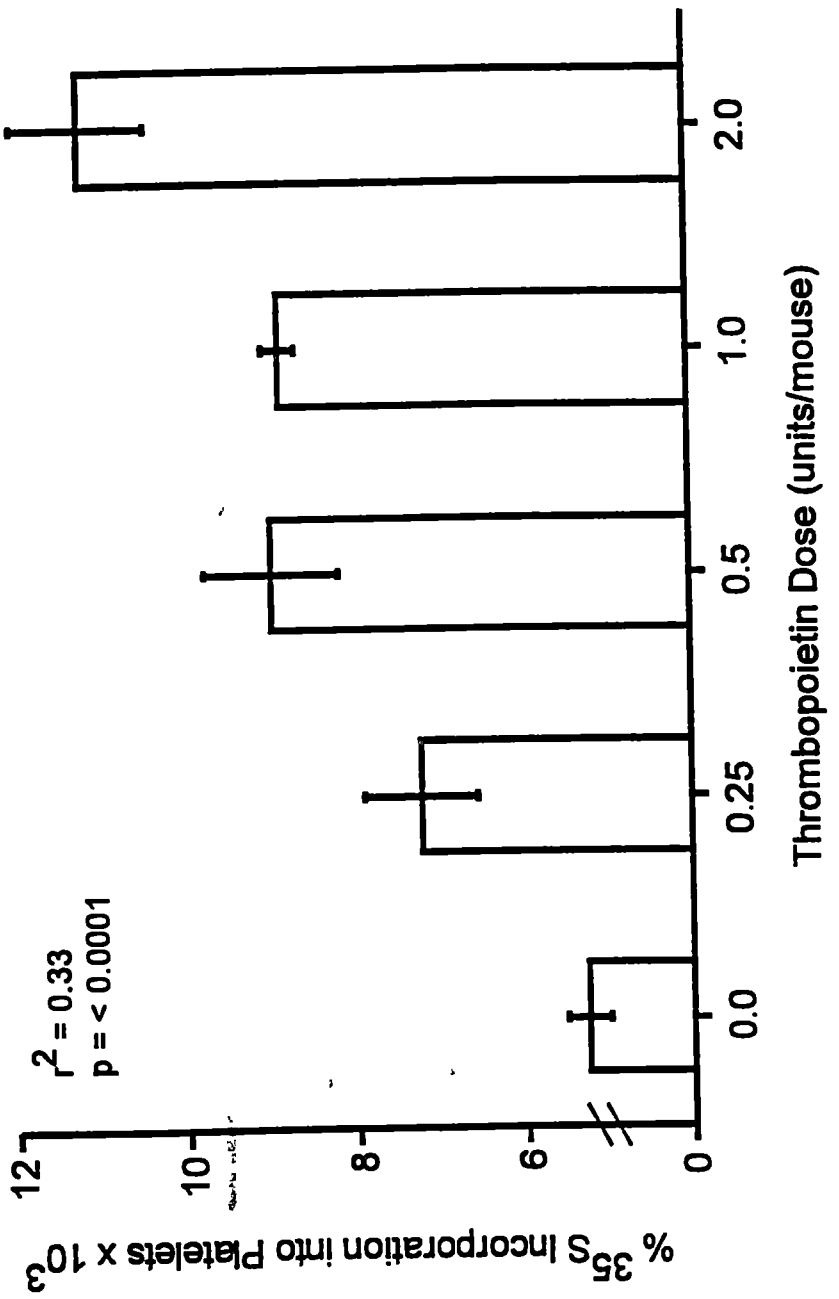
% 35S Incorporation into Platelets x 10^3
12
10
8
6
0
r^2 = 0.33
p = < 0.0001
0.0
0.25
0.5
1.0
2.0
Thrombopoietin Dose (units/mouse)

Figure 2

Figure 3

Linear correlation of dose of thrombopoietin and megakaryocyte ploidy in mice Points indicate mean megakaryocyte ploidy of all mice receiving that dose of thrombopoietin, the vertical bars indicate standard error The total number of mice in the regression analysis was 51

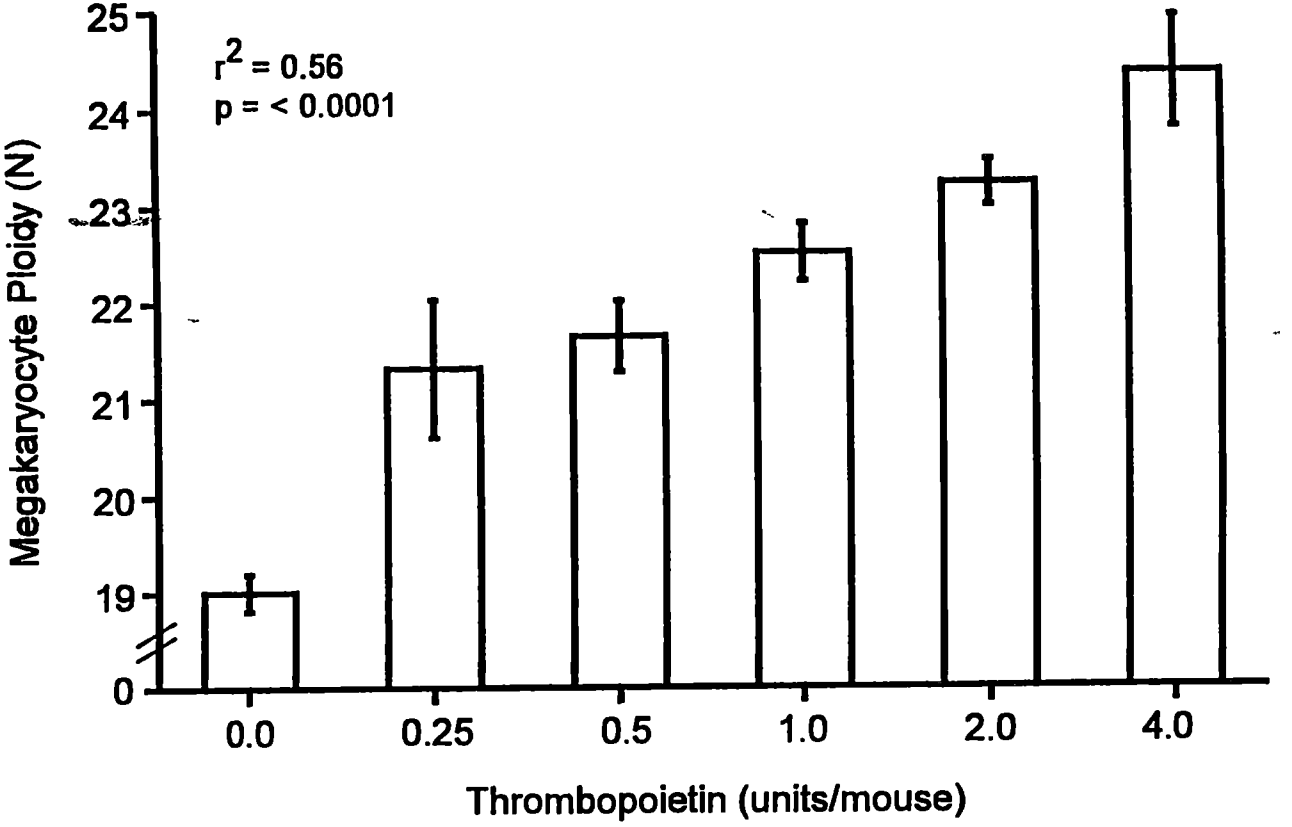
25
24
23
22
21
20
19
0
r^2 = 0.56
p = < 0.0001
Megakaryocyte Ploidy (N)
0.0
0.25
0.5
1.0
2.0
4.0
Thrombopoietin (units/mouse)

Figure 3

Figure 4

Linear correlation of dose of thrombopoietin and packed cell colume in mice Points indicate mean packed cell volume of all mice receiving that dose of thrombopoietin, the vertical bars indicate standard error The total number of mice in the regression analysis was 57

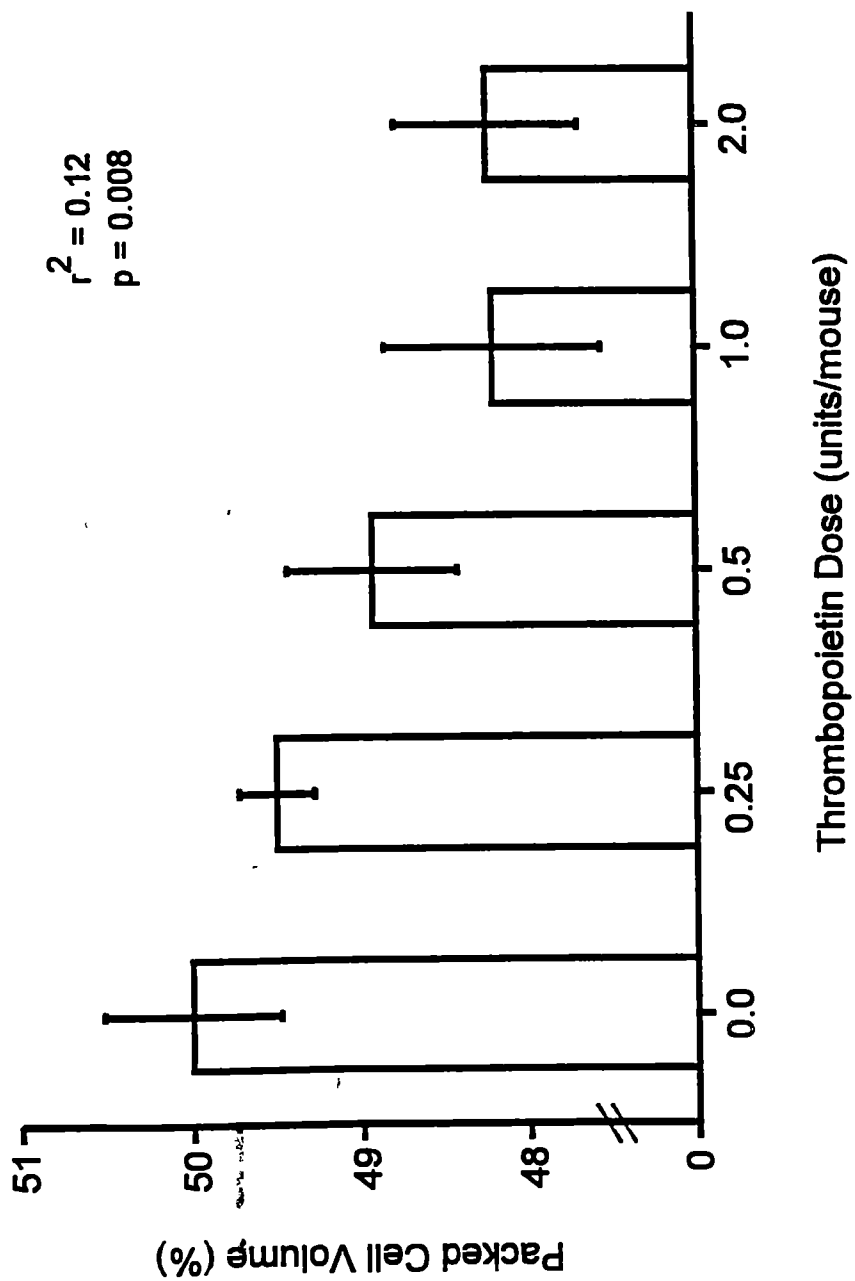
$r^2 = 0.12$
p = 0.008
Packed Cell Volume (%)
51
50
49
48
0
0.0
0.25
0.5
1.0
2.0
Thrombopoietin Dose (units/mouse)

Figure 4

VITA

Patrick Sean Sullivan is the second of two children born to Darcy and Phyllis Sullivan He was born November 28, 1966 in Arlington Heights, Illinois After a family move to Knoxville, Tennessee in 1971, Patrick attended Mount Olive Elementary school and The Webb School of Knoxville, graduating as valedictorian of his class in 1984 From 1984-1988, he attended Emory University as a Woodruff Scholar, where he studied music and biology After graduating with a B S in Biology in 1988, he returned to Knoxville to enter the veterinary curriculum at The University of Tennessee in the fall of that year

Patrick entered the Comparative and Experimental Medicine Program in 1990, and worked concurrently in the Ph D curriculum and in the professional curriculum He graduated with a D V M in May of 1992, and then began full time work in the doctoral program

Upon graduation, Patrick will be travelling to Atlanta, Georgia to accept a post-doctoral fellowship at the Centers for Disease Control in the Division of HIV/AIDS His long term plans include a return to academia, with hopes of a split research, teaching, and clinical appointment