To the Graduate Council:

I am submitting herewith a thesis written by Kendra Lynn Manning entitled "An Investigation of the Effects of Megakaryocyte Ploidy and Spleen Size on Platelet Counts and Platelet Lifespans in Two Strains of Mice: An Animal Model for Studying Hypersplenism." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Comparative and Experimental Medicine.

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An Investigation of the Effects of Megakaryocyte Ploidy and Spleen Size on Platelet Counts and Platelet Lifespans in Two Strains of Mice: An Animal Model for Studying Hypersplenism

A Thesis
Presented for the
Master of Science Degree
The University of Tennessee, Knoxville

Kendra Lynn Manning
May 1996

"After a while you begin to learn the subtle difference between holding a hand and chaining a soul. And you begin to learn that loving doesn't mean leaning and company doesn't mean security. And you begin to know that kisses aren't promises and presents aren't contracts. And you begin to accept your defeats with the grace of a woman and not the grief of a child and learn to build all of your roads today because tomorrow's ground is too uncertain for plans; futures have a way of falling down in midflight. After a while you learn that even sunshine burns if you get too much. So you plant your own garden and decorate your own soul, instead of waiting for someone to bring you flowers. And you learn that you really can endure. That you really are strong. And you learn and learn...With every goodbye you learn."

With love I offer this dedication to the three people that are responsible for preparing me for the challenges of graduate school and beyond: Dr. Ted P. McDonald, Rose Clift, and Marilyn Cottrell. You have truly been my family for the last four years.

In many ways it seems like just yesterday that I was a little freshman knocking on your door looking for a job washing glassware. And now I'm getting my Master's and moving on. I hate to say goodbye.

The three of you have been there to see me change and mature. College was never an easy adjustment for me, but I can definitely say that having the three of you in my life made it so much easier.

The three of you are a great team and I hope that someday I will again work in an environment with so much support, friendship, respect, patience, talent, and integrity. It has been an honor and a joy to work with you. I can't tell you how proud I am to join the ranks of the other graduate students who have come through your lab. "You sure know how to pick 'em!"

I wish you all the best - that's certainly what you gave me!

"To meet, to know, to love - and then to part, is the sad tale of many a human heart."
Samuel Taylor Coleridge

ACKNOWLEDGMENTS

There are many people to whom I am indebted for their support, teaching, guidance, friendship, love, and labors. I am grateful to each and every one of you.

First, I would like to express my deep gratitude to my Committee: Dr. Ted P. McDonald, Dr. Eric Schultze, and Dr. Jan Bright. To Dr. Bright, thank you for dedicating your time, sharing your knowledge, and serving on my committee. To Dr. Schultze, thank you for always being there for my questions and always "checking in" on my progress. I've learned much from you, not just in the area of histology and pathology, but also in grammar and syntax! To my boss turned major professor, Dr. McDonald, I can never thank you enough. I appreciate your patience, guidance, and encouragement during both my undergraduate and graduate careers. You often showed faith in me when I didn't have faith in myself. You showed genuine interest and concern, not only for my work, but also for me as a person. You've made a lasting impression on me. Thank you so much!

There are always those outside the committee who make significant professional and personal contributions, and this is no exception. I will forever be indebted to Rose Clift and Marilyn Cottrell. To Rose, you have been a great friend, teacher, and technician. You are one of the wittiest persons I know and I'm glad you were comfortable enough to cut up with me. You could always make me laugh. You are also an incredible technician and anyone is lucky to have you work with them. I was lucky enough to have you as a teacher. I can't believe the patience you had with me. I bet you thought I would never learn where everything was in the lab (actually, I guess I still have to ask about some things). You taught me how to be an accurate technician and I'll always owe that to you. Thanks for everything! To Marilyn, my adopted Mom, I don't really know what to say. Which is odd, since I was always comfortable talking to you about anything. You're a great listener, a great friend, and a great Mom. Eric and Keith are so lucky to have you! I'll always remember our conversations and our walks. You are the most kind and genuinely caring person I know. I can only hope that some of those characteristics rubbed off on me. Also, thanks for the all the advice and wisdom. I'll carry it with me forever.

Many have assisted in other technical areas as well. Thanks to Betsy Bailey for her help with the FACScan, Doris Millsaps for her help with the Cell-Dyn, and Sherry Cox for the determination of the fluorescence spectra.

Most graciously, I would like to thank my family. To my Mom and Dad, I want to tell you how proud I am to say that I've seen both of you graduate from college. I hope the two of you are as proud of me as I am of you. And to my Grandfather Hansen, thank you for your support, both financial and emotional. I've always appreciated your interest in my classes and projects, although I don't think I'll ever really be able to explain my research to you!

Life, in general, and graduate school, in particular, would not be possible without good friends. I have been fortunate to have some very special ones. I would like to thank April Proud for lending an ear. Special kudos to Keeley Sykes for being one of the greatest friends anyone could ever ask for. I can always say that you added some memorable moments to my college experience. You were always there for me when I needed an ear, a tater tot or KFC, a trip to North Carolina or Cuba, a ride to the airport, and most importantly, when I needed a friend. And to Amy Spann, my great friend and long time neighbor, thanks for the many hours of talks. I'll never understand how I can talk to you for hours, even though I just talked to you the day before. You are the greatest sounding board in the world and you also give wonderful advice. I wish you the best of luck in your graduate program. I'm really proud of you!

Lastly, I would like to thank David Grant. I believe that I have truly found my soulmate in you. I couldn't have made it through the last year and a half without you, and even if I could have, it wouldn't have meant as much. You've provided me with more love, support, and encouragement from 400 miles away than I ever thought possible. I hope our paths will cross again soon.

ABSTRACT

Several studies have shown that C3H mice have a modal megakaryocyte ploidy class of 32N instead of 16N that is found in all other strains of mice that have been tested [1,2]. In addition to greater amounts of DNA in their megakaryocytes, C3H mice have greater platelet production, but lower platelet counts than C57BL mice. The work reported herein was undertaken to determine the cause of the mild thrombocytopenia in C3H mice. In agreement with the results of increased percent ^{35}S incorporation into platelets [3], several previous studies have shown increased platelet production in mice with elevated ploidy megakaryocytes [4-6]. Hematological studies, including platelet counts, packed cell volumes (PCV), white blood cell counts, white blood cell differential counts, and platelet lifespans, in addition to serum testosterone levels, were determined on both C3H and C57BL mice. C57BL mice were used as the control mouse strain, representing mice with 16N modal ploidy megakaryocytes. Because of the marked visual difference in the spleens of the two strains of mice, spleens were weighed and examined histologically and splenic megakaryocyte size and number were determined. In addition, tissue weights of the liver, lung parenchyma, and kidneys were determined for the two strains of mice. As a follow up, other C3H and C57BL mice were splenectomized for determination of the role of the spleen in decreasing the platelet counts of C3H mice as compared to C57BL mice.

The data of the present study, in agreement with previous studies [1,4,7-9], show that C3H mice had significantly lower platelet counts ($p<0.0005$) and PCV's ($p<0.0005$) than C57BL mice. Also, an unexplained observation was that C3H mice had higher

($p<0.005$) serum testosterone levels than C57BL mice. In addition to having larger spleens ($p<0.0005$) than C57BL mice, C3H mice have spleens that contained larger and more numerous megakaryocytes ($p<0.005$), indicating a possible compensatory mechanism for correction of the mild thrombocytopenia. We also determined that C3H mice had a platelet lifespan of 4.8 days, which is significantly shorter ($p<0.01$) than the 5.7 day platelet lifespan than was found for C57BL mice. When splenectomized, platelet lifespans for C3H mice were significantly ($p<0.005$) longer than that found in intact C3H control mice, but in the C57BL mice, splenectomy had no effect on platelet lifespans ($p>0.40$). The lengthening of platelet lifespans for C3H mice by splenectomy lends credence to the hypothesis that the spleens of C3H mice were sequestering or destroying platelets at a faster rate than found in C57BL mice. Moreover, C3H mice had larger spleens with evidence of the white pulp expansion, larger lymphoid nodules, and greater amounts of periarterial lymphatic sheaths. These data support the hypothesis that hypersplenism in C3H mice results in the mild thrombocytopenia with increased platelet production compared to values from C57BL mice.

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CHAPTER I.

INTRODUCTION

Most inbred strains of mice, including C57BL mice, have 16N as the modal megakaryocyte ploidy [1]. However, it was found, quite unexpectedly, that C3H mice have on average twice the DNA content in their megakaryocytes as other mice strains, i.e., C3H mice have been shown to have 32N as the modal megakaryocyte ploidy [1,2]. In agreement with the finding of higher modal ploidy megakaryocytes in C3H mice, larger megakaryocytes have also been found, but a lower concentration of these cells are present in the bone marrow of C3H mice compared to other mouse strains, including C57BL mice [1,4,7]. Upon further investigation of C3H mice, it was shown that C3H mice have lower platelet counts than C57BL mice [1,4,7]. However, C3H mouse platelets have increased percent ^{35}S incorporation, which provides evidence that they have increased platelet production compared to values from C57BL mice [3]. It was also shown that male mice, regardless of strain of mice, have significantly higher platelet counts and megakaryocyte ploidy than female mice of the same strain [1,3,7]. The author hypothesizes that the difference in platelet count and megakaryocyte ploidy can be explained by the male mice having higher testosterone levels than the female mice. Although the platelet sizes of both strains of mice appear to be the same [10], the platelets of C3H mice were not as functional as platelets from other mouse strains, including C57BL mice, as shown by sensitivity to aggregating agents [11]. Therefore, there must be a difference in the structure or content of C3H mouse platelets that would make them less responsive to aggregating agents as compared to C57BL mice.

Traditionally, it was believed that higher ploidy megakaryocytes produce more platelets than low ploidy megakaryocytes [12,13]. However, C3H mice have higher ploidy megakaryocytes and lower platelet counts than other strains of mice, but evidence of increased platelet production in C3H mice compared to platelet production in C57BL mice has been demonstrated [3]. The work presented here is an attempt to explain the differences in the platelet counts, in spite of the differences in average megakaryocyte ploidy and platelet production, of C3H and C57BL mice. To investigate the cause of this anomaly, we studied several indicators of platelet production, such as platelet count and platelet lifespan, in these two strains of mice. Several hypotheses have been advanced to explain this anomaly (deviation from the common type). One theory that we have observed for this finding, although unlikely, is that C3H mice have a lower testosterone level than C57BL mice (since C57BL mice are observed to be more aggressive) and, therefore, lower platelet production [3]. Although previous data [3,4], which showed elevated percent ^{35}S incorporation into platelets of C3H mice as compared to C57BL mice, disagree with this hypothesis; these workers [3] advanced several possible explanations for the higher percent ^{35}S incorporation seen in C3H mice. First, C3H mouse platelets may contain a higher level of sulfated proteoglycans or mucopolysaccharides. Therefore, sulfated groups on the surface of C3H mouse platelets would incorporate more ^{35}S label. Second, clearance of ^{35}S sulfate may be slower in C3H mice as compared to C57BL mice. Finally, the higher megakaryocyte ploidy may cause an increase in the uptake of the sulfur label.

Another explanation for the lower platelet counts is that the platelets of C3H mice may have an elevated amount of mucopolysaccharides on their surface, which could make them more likely to either be sequestered or destroyed by the spleen. Several lines of investigation indicate that this could be true, i.e., platelets of C3H mice have increased ^{35}S incorporation [3] and C3H mice have decreased platelet counts compared to values from C57BL mice [1,4,7]. Therefore, our final hypothesis is that the spleens of C3H mice may be hyperactive and sequestering more platelets than normal. The sequestration may also cause the platelet lifespans of the C3H mice to be shorter than values from C57BL mice.

Hematological studies were performed on both C3H and C57BL mice, along with testosterone levels to determine the reason for the different platelet counts. In addition, some mice were splenectomized to determine the role of the spleen in lowering platelet counts of C3H mice. Spleens were weighed, examined histologically, and their splenic megakaryocytes analyzed. The platelet lifespans of intact and splenectomized C3H and C57BL mice were also determined using *in vivo* biotinylation, which is a sensitive assay for measurement of platelet lifespans.

The present study confirms previous work [1,4,7-9] and shows that C3H mice have lower platelet counts and lower packed cell volumes than C57BL mice. Although unexplained, we report that C3H mice have higher testosterone levels than C57BL mice. Larger and possibly more active spleens, and larger and greater numbers of splenic megakaryocytes were found in C3H mice compared to values from C57BL mice. Importantly, C3H mice also have a shorter platelet lifespan compared to values of C57BL mice; the platelet lifespan of splenectomized C3H mice was longer than that found in

intact C3H control mice, indicating an important role for the spleen in decreasing platelet counts of these mice. An additional important observation is that platelet lifespans were not altered by splenectomy in C57BL mice.

The findings of this work indicate that C3H mice not only have a higher modal megakaryocyte ploidy than C57BL mice, but that C3H mice also have lower platelet counts with evidence of elevated platelet production compared to C57BL mice. C3H mice have a shortened platelet lifespan as compared to C57BL mice that is restored to control values with splenectomy. Moreover the spleens from C3H mice are larger, and, perhaps, more active than C57BL mice, which could lead to either sequestering more platelets or destroying them at a faster than normal rate (hypersplenism). We believe this increased splenic activity causes a shortened platelet lifespan in the C3H mice, which leads to lower platelet counts than other mice with 16N modal megakaryocyte ploidy (C57BL). We believe that C3H mice would be a suitable animal model for studying hypersplenism.

CHAPTER II.

REVIEW OF THE LITERATURE

Introduction. The basic purpose of this study was to determine why mice with higher modal ploidy megakaryocytes have significantly lower platelet counts, since it has been considered dogma that high ploidy megakaryocytes lead to elevated platelet counts [13-15]. In support of the finding of lower platelet counts, mice with higher modal megakaryocytes also have lower total circulating platelet counts (TCPC), but increased percent ^{35}S incorporation, increased megakaryocyte diameter with decreased megakaryocyte number, and increased total megakaryocyte mass (TMM) [3,7]. It has been reported that most strains of mice, including C57BL mice, have 16N modal ploidy megakaryocytes [1-4,7,10,16]. Completely by accident, it was discovered in our laboratory in 1988 that C3H mice have a modal megakaryocyte ploidy class of 32N [2]. This discovery was important for several reasons. It was the first strain of mice to be identified that had a modal megakaryocyte DNA content that was different from all other mouse strains. This finding has led us to focus on C3H mice as a tool in the understanding of the role of ploidy on both megakaryocytopoiesis and thrombocytopoiesis.

This chapter presents a review of the literature and research related to this study. The chapter begins with a discussion of the megakaryocytes, including megakaryocyte ploidy and the influence of ploidy on platelet production and megakaryocyte size and number. Next, the role of genetics and genomic imprinting on megakaryocyte ploidy and platelet production are discussed. This is followed by an overview of the influence of sex of the mouse on megakaryocytopoiesis and thrombocytopoiesis. The next section of this

review proposes several hypotheses for the observed lower platelet counts in mice with higher modal ploidy megakaryocytes. The review is concluded with a summary of the known differences between C3H and C57BL mice.

Megakaryocytes. Megakaryocytes, the large polyploid cells found in the bone marrow and spleen that are responsible for platelet production, are different from other cells in that they have a unique development process. This process involves DNA replication without cell division (cytokinesis) followed by cytoplasmic maturation and platelet production. Megakaryocytes, which are found in the bone marrow and spleen, originate from an uncommitted, multipotential stem cell that can, when induced, differentiate into a committed precursor cell [17]. This step, which is very slow under normal conditions and faster under conditions of high platelet demand, begins the megakaryocyte differentiation process. Once diploid cells (2N) are committed, cellular proliferation is followed by nuclear proliferation and mitotic amplification of megakaryocyte precursor cells. These steps are followed by further nuclear proliferation and endomitotic nuclear division. After endomitosis begins, megakaryocyte cytoplasmic growth proceeds with the acquisition of platelet-specific proteins and cell organelles [18]. After reaching one of several degrees of polyploidization, the megakaryocytes cease to replicate DNA and undergo the final differentiation period [18,19].

Recognizable megakaryocytes undergo a series of maturational changes in the cytoplasm which have been called "stages". These stages are referred to as stages I, II, and III, with stage I representing the immature megakaryocytes of all ploidy classes (i.e., 8N, 16N, 32N, and 64N) [17]. In the mature cell, ploidy is characterized by the presence

of four, eight, or sixteen nuclear lobes. The classes are termed 8N, 16N, 32N, and 64N in comparison with a diploid (2N) precursor cell. Therefore, it takes two cycles to go from the committed diploid cell to a recognizable 8N megakaryocyte, three for 16N, etc. Stage II megakaryocytes are formed by further cytoplasmic maturation. At this stage, more platelet-specific proteins are acquired [17]. Finally, stage III megakaryocytes are produced, followed by formation of microvesicles in the cytoplasm which lead to platelet demarcation membranes. The vesicles pinch off and platelets are released into the circulation [17]. Shedding of platelets in the production process leads to the beginning of the appearance of naked nuclei of all ploidy classes in the marrow [19].

One of the unusual characteristics of the cytoplasmic structure of the megakaryocyte is its demarcation membrane system (DMS). In a normally mitotic diploid cell, there is an increase in the synthetic activity of the cell that is responsible for the production of enough membrane for two daughter cells. However, in the endomitotic megakaryocyte, every endomitosis is associated with a stimulus for membrane formation, but without cell division. It has been shown that the DMS is, itself, the analog of the plasma membrane and is invaginated from the cell's surface [20]. Polyploidy in megakaryocytes is, therefore, best viewed as a form of cellular specialization related to the remarkably prolific membrane formation. It has also been shown that the higher the ploidy of a megakaryocyte, the greater the content of membrane in the cytoplasm [5]. Thus, it is believed that increased ploidy of megakaryocytes leads to increased platelet production, and thus platelet counts [4,13-15]. Also, the concentration of granules and mitochondria in the cytoplasm of megakaryocytes varies inversely with membrane content, and

comparing cells of each ploidy class, there is a significant difference between the cytoplasm of 8N, 16N, and 32N cells at the granular megakaryocyte stage [5,21].

The DMS is known to provide the surface membrane of the circulating platelet and its surface canalicular system [20,22]. The membrane may play an important role in platelet lifespan and possibly function. Therefore, it is tempting to speculate that platelets produced from different ploidy megakaryocytes are different in regard to their membrane surface and canalicular system.

It is now widely accepted that platelet number, mass, and function are the major regulatory influences of megakaryocytopoiesis [17] and, thus, thrombocytopoiesis. Increases in megakaryocyte size [23,24] and number [12,25] have been found to be good indicators of the level of megakaryocytopoiesis. There is also evidence that demonstrates that there is autoregulation of megakaryocytopoiesis by acetylcholinesterase released from mature megakaryocytes [26-28]. It is also recognized that a hormone primarily produced in the liver and kidney, thrombopoietin, is a major regulator of megakaryocytopoiesis and, thus, thrombocytopoiesis [29,30]. Investigations have shown that platelets themselves bind thrombopoietin, which may provide a feedback mechanism for thrombocytopoiesis [31-34]. However, this feedback can not be the only regulation of platelet production because platelets made nonfunctional by means such as acetylsalicylic acid (ASA or aspirin) do cause an increase in thrombopoietin levels and, therefore, exhibit an increase in platelet production [35]. Acetylsalicylic acid inhibits platelet function by inhibiting prostaglandin synthetase with impaired thromboxane A_2 production. Therefore, the

binding of thrombopoietin to platelets cannot be the only means of regulating platelet production.

Several studies have shown that thrombopoietin injected into mice will increase megakaryocyte size and number [36], markedly improve the number of small acetylcholinesterase positive (SAChE+) cells [37], elevate the rate of megakaryocyte endomitosis [38-40], and increase megakaryocyte maturation [41]. Thrombopoietin also has a positive effect on the number of megakaryocytic colonies in the spleen of bone marrow reconstituted mice [42].

SAChE+ cells are early cells in the megakaryocytic series and appear to be sensitive indicators of changes in platelet production [37]. If exogenous sources of thrombopoietin are given in increasing amounts, there appears to be a significant increase in the number of SAChE+ cells present in the bone marrow [37]. Also, an inverse relationship between the platelet counts and the percent of SAChE+ cells was found after injections of rabbit anti-mouse platelet sera (RAMPS), further demonstrating the role of platelet number and mass as a regulator of megakaryocytopoiesis [37]. Therefore, thrombopoietin acts on both mature cells and on an early cell of the megakaryocytic lineage that leads to an increased number of SAChE+ cells.

Megakaryocyte Anomaly. Several studies have shown that C3H mice, which have higher ploidy megakaryocytes, also have lower platelet counts than other mouse strains [1,3,4]. However, platelet size does not appear to be related to the ploidy of the megakaryocyte from which it was released [3,10]. Platelet size seems to be determined more by feedback demand by the system than by the difference in the ploidy of the

megakaryocytes. Under high platelet demand conditions, such as severe thrombocytopenia, all ploidy classes appear to release platelets. In high demand conditions such as these, younger platelets also have increased ^{35} S incorporation into platelets.

It has been shown that there is an apparent transfer of ^{35}S label from the golgi apparatus of the megakaryocyte to the DMS, which suggests there are sulfated compounds being produced by the golgi apparatus [21]. These compounds that are being labeled are mucopolysaccarides in the membrane. Therefore, platelets that originate from different ploidy megakaryocytes may have different mucopolysaccharides on their surfaces. This is in agreement with earlier investigations that show that megakaryocytes of different ploidy classes are labeled differently [43]. It has been shown that newly produced platelets have increased ^{35}S incorporation compared to platelets that have been in circulation for several days [44]. The ^{35}S label is incorporated into newly formed platelet proteoglycans and is not reutilized if given as sodium sulfate [45]. Many studies have shown that thrombopoietin increases the percentage of ^{35}S-sodium sulfate incorporation into platelets of mice [46-48]. The isotope incorporation into platelets appears to be a linear thrombopoietin dose-dependent relationship [29]. It has also been shown that C3H mice have a greater percent ^{35}S incorporation into platelets than values from other strains of mice [49], indicating elevated platelet production.

Genetics. Studies have been performed recently on the mode of inheritance of the 32N modal megakaryocyte class in C3H mice [3,7,50]. To do this, backcross matings of both C3H and C57BL mice were made and the DNA content of the offspring's

megakaryocytes examined [7]. The polyploid DNA content of the megakaryocytes from the offspring confirmed previous work, showing that C3H mice have higher percentages of higher ploidy megakaryocytes than did the other mice. Also, the offspring with the largest amount of DNA in their megakaryocytes had the lowest platelet counts [3,7]. It was also shown that with increasing amounts of C3H mouse genetics, there was a significant decrease in platelet count. This theory is further supported by evidence that shows that the frequency of higher ploidy megakaryocytes among the offspring of backcross matings was correlated to the proportion of C3H genetics in the offspring [7].

Also, the megakaryocyte DNA distribution of each individual backcross showed continuous distribution [7]. These findings have led to the conclusion that the higher megakaryocyte DNA content of C3H mice is controlled by multiple alleles.

Genomic Imprinting. It has also been shown that genomic imprinting plays a role in the determination of modal megakaryocyte DNA content [3,7,50]. Genomic imprinting is defined as the difference in expression of genes depending on whether the genetic material was contributed by the female or male parent. In the case of megakaryocyte DNA content, it has been shown that the maternal parent contributes more to the genotype than the paternal parent [7]. This work also shows that the female's DNA contribution to the offspring had a greater effect on the megakaryocyte ploidy and, therefore, platelet production than did the male's contribution. Therefore, C3H mice may be contributing a larger dosage of genes to their offspring than other strains of mice which would result in the C3H mouse offspring having higher average megakaryocyte DNA content.

Sex of Mice. It is well known that testosterone has many effects on hematopoiesis in mice, e.g., testosterone has been shown to increase red blood cell production [51]. Although there have been fewer studies to determine the effect of testosterone on platelet production, recent convincing data show that testosterone increases thrombocytopoiesis. This work has shown that intact male BALB/c mice have higher platelet counts than either female or castrated BALB/c mice [8,9,52,53]. Castrated BALB/c mice, compared to intact male BALB/c mice, have lower body weights, packed cell volumes, total red blood cell circulating masses, total circulating red blood cell counts, platelet counts, sulfur 35 incorporations, mean megakaryocyte ploidies, total circulating platelet masses (TCPM), and total circulating platelet counts (TCPC) [53]. Administration of testosterone to castrated male BALB/c mice returned these hematological values back to values of intact male BALB/c mice. However, it has also been shown that male and female mice of the same strains show the same amount of sensitivity to thrombopoietin [49]. Moreover, in C3H mice, megakaryocyte number are the same in males and females [1,3], but as yet unexplained the males have higher average ploidy megakaryocytes and, therefore, larger megakaryocytes than the female C3H mice [1]. In agreement with the data on BALB/c mice, intact male C3H mice also have higher platelet counts than castrated C3H mice [8]. Investigations have shown that with offspring from crosses and backcrosses of C3H and C57BL mice, with different genetic makeup, male mice had significantly higher platelet counts and megakaryocyte ploidy than the females mice of the same genetic makeup [3,7]. Therefore, it can be shown that testosterone levels affect

platelet production in mice, but additional factors cause the difference in platelet production between genders.

Hypotheses. Although megakaryocytes are found in the spleen, the spleen is primarily an erythropoietic organ [54]. However, in times of severe thrombocytopenia, such as bone marrow ablation, the spleen may produce significant numbers of platelets [55]. In addition, it has been shown that splenectomized mice show an increase in platelet counts and a decrease in packed cell volumes compared to intact mice [54-58]. It has also been demonstrated that thrombopoietin will increase the number of megakaryocytic colonies in the spleens of lethally irradiated, bone-marrow-transplanted mice [29,42]. The size and number of splenic megakaryocytes has also been evaluated and it was shown that the megakaryocytes of spleens do not differ in size from those found in the bone marrow [59] and that under hypoxic conditions the size and number of splenic megakaryocytes decrease [59,60]. Changes in megakaryocyte diameter and number with hypoxia suggest a compensatory mechanism for increasing platelet production, which may be regulated separately in the spleen and bone marrow [55]. However, it remains to be determined if splenic megakaryocytes from C3H mice are different from the splenic megakaryocytes of C57BL mice. In addition, it has been shown that splenic enlargement is associated with the depression of platelet counts [58]. Therefore, C3H mice may have larger spleens than C57BL mice, which could be causing the mild thrombocytopenia.

It was proposed that platelets released from 32N megakaryocytes would be unusually sensitive to changes in the plasma because of the liberal content of an open tubular system [61]. This agrees with the findings that C3H and C57BL mice have

significantly different responses to various aggregating agents [11]. Because C3H mice are the more resistant to atherosclerosis and C57BL mice are the more susceptible, platelets from the two strains of mice have been used in aggregation studies [11]. It was found that platelets from male mice are more sensitive to aggregating agents than are platelets from females of the same strain. It was also shown that platelets from C3H mice have an impaired response to arachidonic acid and ADP, yet these platelets showed an increased response to ristocetin as compared to platelets from C57BL mice. It was shown that the abnormality in the platelets from the C3H mouse is clearly a platelet defect and not a plasma derived defect [11]. Although a difference in platelet aggregation exists between platelets from the two stains of mice, this does not completely explain the difference in the mouse's resistance to atherosclerosis. However, these studies show that the surface of the platelets from C3H mice differ from the platelet surfaces of C57BL mice. One possible explanation for these findings is that C3H mice have more mucopolysaccharides in the membranes of their platelets than that found in platelets of C57BL mice, which could be linked to the difference in aggregation. Although there is no evidence, the mucopolysaccharides could make the platelets "sticky" and, therefore, help in the aggregation response.

Blood platelet research programs frequently use mice, such as C3H and C57BL mice, as test animals. However, platelet lifespans in these animals are not easily measured. Platelet lifespans of mice have been determined using labeling with radioisotopes [62-65]. Non-radioactive methods for determining murine platelet lifespans have utilized

fluorescent lipophilic membrane probes [66], mouse monoclonal antibodies [67], and more recently *in vivo* biotinylation [68].

Results of platelet lifespans obtained by the use of ^{111}In in BALB/c mice gave lifespans of approximately 1.83 - 2.40 days [63,64]. However, results from earlier work using ^{35} sulfur, ^{51} chromium, and ^{54} chromium reported a platelet lifespan for BDF_1 and C3H mice of about 4 days [62,65,69]. Studies of the lifespan of reinfused platelets in mice have shown the platelet lifespan to be approximately 4.5 days [66]. Recent work using biotinylation in mice to determine the age of certain sub-populations of platelets reported the platelet lifespans of outbred Swiss-Webster mice to be 4.5 days [70]. A difference in platelet lifespans of C3H and C57BL mice could possibly explain the mild thrombocytopenia seen in C3H mice.

Conclusion. To summarize, C3H mice have a higher modal megakaryocyte ploidy and lower platelet counts than most other strains of mice, particularly C57BL mice. In addition, the two strains of mice differ in many ways: C3H mice have greater megakaryocyte sizes, serum testosterone levels, neutrophil counts, and percent ^{35}S incorporations into platelets, but have lower PCV's, TCPC, megakaryocyte numbers, and their responses to aggregating agents is reduced compared to values from C57BL mice. Another finding of the present work is that C3H mice have larger spleens which contain larger and more numerous megakaryocytes than C57BL mice. We also report herein that C3H mice have a shortened platelet lifespan as compared to C57BL mice which is returned to normal values with splenectomy.

CHAPTER III.

MATERIALS AND METHODS

Animals. Male C3H/HeNHsd (C3H) mice and male C57BL/6NHsd (C57BL) mice, 4-5 weeks of age, were purchased from Harlan Sprague-Dawley (Indianapolis, IN). Mice were allowed to acclimate in The University of Tennessee College of Veterinary Medicine's animal facility for approximately two weeks before being involved in any procedures. The animal protocols were approved by The University of Tennessee's College of Veterinary Medicine Animal Care and Concerns Committee.

***In vivo* biotinylation.** Prior to bleeding, male C3H and C57BL mice, 6-8 weeks of age, were injected intravenously (iv) with Sulfo-NHS-Biotin (Pierce, Rockford, IL) at a dose of 35 mg per kg of body weight administered in 0.5 ml of sterile normal saline. This dosage of biotin is approximately the same as that used successfully in dogs [71]. Four experiments were performed. In the first two experiments, mice were 6 weeks of age at time of injection and the intact and splenectomized mice in the third and fourth experiments were 7 weeks of age at time of biotin injection.

Collection and preparation of blood samples. At 24-hour intervals following injection of Sulfo-NHS-Biotin, groups of two to four mice were killed humanely. Approximately 10 minutes before killing, mice were given an intraperitoneal (ip) injection of 0.5 ml heparin-pentobarbital-saline solution (1 ml heparin [1000 U.S.P. units], 1 ml pentobarbital [50 mg], 10 ml saline) [72]. Mice were bled from the retroorbital sinus to obtain blood for determination of platelet counts, packed cell volumes (PCV), and testosterone levels. Mice were then bled from the heart into 3 ml syringes containing 1 ml

of disodium ethylenediaminetetraacetic acid (EDTA) solution (1% EDTA + 0.538% NaCl). Approximately 0.5 ml of blood was collected from each mouse and expressed into a 12 x 75 mm polystyrene tube.

Platelet Counts. Platelet counts were determined by manually diluting a sample of blood 1:100 into a red blood cell pipette with filtered 1% ammonium oxalate (AO) to lyse red blood cells. Samples were then placed in a hemacytometer chamber, and platelets were counted manually using phase microscopy.

Packed Cell Volumes. Blood was drawn from the retroorbital sinus into heparinized capillary tubes, the ends were sealed with Seal-Ease (Clay-Adams, Inc., New York, N.Y.), tubes were centrifuged for 5 minutes in a micro-hematocrit centrifuge (International Equipment Co., Needham Heights, Mass.), and the percentage of packed cells was measured using a micro-capillary reader (International Equipment Co., Needham Heights, Mass.).

Testosterone Levels. Serum collected from blood obtained from the retroorbital sinus of intact mice was used in an ^{125} I-testosterone radioimmunoassay (RIA)(ICN Biomedicals, Inc.) to determine the serum testosterone levels. Competitive inhibition RIA measured the concentration of antigen (testosterone) in the sample by determination of the extent to which the testosterone combines with the testosterone antibody. In this assay, serum samples are added to a tube, followed by a known amount of ^{125} I-testosterone, followed by a limited amount of antibody specific for testosterone. The mixture is then incubated. With increasing amounts of testosterone in the test sample, a correspondingly decreased fraction of ^{125} I-testosterone was bound to the antibody. After separation of the

bound testosterone from the free testosterone by the use of a 2nd antibody and centrifugation, the amount of radioactivity in the precipitate was evaluated and compared to a standard curve to determine the amount of testosterone in the serum samples from the C3H and C57BL mice.

Preparation of platelet samples. For platelet sample preparation, blood diluted in EDTA was centrifuged at 160 x g for 4.5 minutes to allow collection of platelet-rich plasma (PRP), and the PRP was centrifuged at 800 x g for 15 minutes to obtain a platelet pellet. Platelet pellets were washed once with 0.6 ml and once with 0.5 ml AO to lyse all remaining red blood cells. After washing with AO, platelets were washed with 1.5 ml saline to remove the residual AO. The platelets were resuspended in 0.4 ml phosphate-buffered saline (PBS), pH 7.2, followed by the addition of 0.1 ml R-phycoerythrin conjugate streptavidin (Molecular Probes, Inc., Eugene, OR) that had been diluted 1:20 with PBS. Platelet suspensions were incubated on a mixer with a rocking motion at 37°C for 30 minutes and then washed twice using 1 ml "standard azide buffer" (SAB) [73] with centrifugations of 800 x g for 15 minutes. The SAB in the second wash was previously filtered through a 0.2 μm pore size filter. Platelets were then resuspended in 0.1 ml filtered SAB plus 0.1 ml 2% paraformaldehyde solution and stored at 4°C. Flow cytometry on all samples was performed at the conclusion of each experiment.

Flow cytometry. Emission and excitation fluorescence spectrum of a representative platelet sample were determined using a Shimadzu RF5000U spectrofluorophotometer (Shimadzu, Kyoto, Japan). Emission was found to be maximal at 579.2 nm, thus the fluorescence flow cytometer was adjusted accordingly. Platelet

samples were analyzed by use of a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) equipped with an argon laser. Laser emission was at 488 nm and phycoerythrin (PE) fluorescence was detected using a 585/42 nm band pass filter. The instrument was calibrated for forward light scatter using 2.02 μm diameter latex beads (Coulter Corp., Hialeah, FL) and the fluorescence was calibrated using Calibrite flow cytometer beads (Becton Dickinson Immunocytometry Systems, San Jose, CA). Isotonic-buffered saline (Baxter Healthcare Corp., McGraw Park, IL), filtered through a 0.2 μm pore size filter, was used as the diluent. Platelets were identified by forward light scatter versus side light scatter and 10,000 particles (events) were measured for each sample. On the FACScan, fluorescence signals were collected using 4 decade logarithmic amplifiers represented by a linear scale of 256 channels. The Consort 30 software package was used to capture data generated by the FACScan. All parameters were collected and stored in list mode files and were later analyzed with the Consort 30 software package.

Data collection. Assuming that initially all platelets were irreversibly labeled *in vivo* with Sulpho-NHS-Biotin, we used gates on the flow cytometer to distinguish between labeled platelets and newly produced (unlabeled) platelets. Gates of the list mode files were determined using histograms of PE fluorescence of samples from Day 1. Gates were placed around the peak of PE fluorescence. Figure 1* shows examples of gates for fluorescence histograms at Days 1 and 4 for C3H mice. Using the same gates throughout, percentages of gated events for all samples were then recorded. In the data reported here, "percent gated events" refers to the proportion of platelets that have

* All tables and figures may be found in the Appendix.

fluorescence above a background level. The background fluorescence level was determined using samples derived from control mice given an iv injection of normal saline.

Splenectomy. A representative sample of both strains of mice were splenectomized two weeks before being placed in experiments. Mice were anesthetized by inhalation of Metofane (Pitman-Moore, Mundelein, IL). The area was aseptically prepared before a small incision was made on the left side of the abdomen. The spleen was then excised and the peritoneum and skin were sutured with 4-D silk sutures. After surgery, mice were placed on a sterile, heated field for recovery. Both intact and splenectomized mice were administered Sulfo-NHS-Biotin using identical protocols described above, and platelet counts, PCV's, and platelet lifespans were determined.

Splenic Megakaryocyte Size and Number. Since preliminary experiments showed that C3H mice had larger spleens than C57BL mice, we investigated the splenic megakaryocytes from C3H and C57BL mice. The spleens from 5 C3H and 5 C57BL untreated mice were excised and placed in 10% neutral-buffered formalin. To enhance fixation, the spleens were bisected sagitally and processed in a Vacuum Infiltration Processor (Miles Laboratory, Inc., Elkart, IN). After being processed, the spleens were embedded in paraffin, sectioned on a microtome (Vashaw Scientific Incorp., Atlanta, GA) to a thickness of 5 μm at 100 μm intervals, and stained with hematoxylin and eosin.

Megakaryocyte size and number were obtained from splenic tissue using stereological techniques described by Cullen and McDonald [74]. The areas of 50-200 megakaryocyte profiles were measured at a magnification of 1200x via video overlay onto an x-y digitizing tablet of the Micro-Tech 100 Morphometric and Densometric Image

Analyzing System (Analytical Imaging Concepts, Irvine, CA). By treating the megakaryocytes as spheres, the area of each profile was used to calculate its diameter: $d = 2 \times [\text{square root of } (\text{area}/\pi)]$.

Upon determination of the megakaryocyte profile, the profiles were grouped according to size. Once, all the megakaryocyte profiles were determined, a histogram from the profile frequency was determined. The megakaryocyte profiles that were too small to be counted and the optically lost profiles were estimated using previously established techniques [74]. The frequency distributions were then used to calculate the mean diameter of section profiles (d) which was then used to calculate the individual megakaryocyte diameter (D): $D = 4 \times d/\pi$. The number of section profiles per unit area (N_A) and D were used to calculate the number of spherical particles per unit volume (N_V) while taking into account the thickness (t) of the tissue: $N_V = N_A/(D + t)$.

Tissue Weights. Spleen, liver, lung parenchyma, and kidneys, harvested from the previously bled mice, were weighed. Weights were expressed as a percent of body weight.

White Blood Cell Counts and Differential Counts. WBC differential counts were performed on untreated mice of both strains using both a Cell-Dyn 3500 (Abbott Diagnostics, Abbott Park, IL) and manual techniques. Absolute white blood cell counts were only determined using the Cell-Dyn 3500.

Statistics. Calculations were done using a commercially available statistical package (SAS, SAS Institute, Cary, NC). Two regression models were performed on each of the experiments. The first model used log-transformed data, and the second model

used non-transformed data (simple linear regression). The exponential model did not provide as good a fit as simple linear regression, so results of linear regression are reported herein. Statistical comparisons were made using Student's t-test for unpaired data [75]. Samples were tested at a significance level of $p < 0.05$. All data are presented as means for each day or strain $\pm$ the standard error of the mean.

CHAPTER IV.

RESULTS

Platelet Counts. Figure 2 compares platelet counts of C3H and C57BL mice. In agreement with previous studies [1,4,7], the results of the present study show that C3H mice had significantly lower platelet counts than did C57BL mice ($p < 0.0005$).

Packed Cell Volumes. Packed cell volumes are shown in Figure 3. As shown, C3H mice had significantly lower packed cell volumes than C57BL mice ($p < 0.0005$).

Testosterone Levels. Results of ^{125} I-testosterone radioimmunoassay show that serum testosterone levels (Figure 4) were significantly higher in C3H mice than in C57BL mice ($p < 0.005$).

Tissue Weights. In agreement with the author's visual observations, C3H mice had significantly larger spleens as a percentage of body weight than C57BL mice (Figure 5) ($p < 0.0005$). In addition, the weights of other tissues (expressed as mg per g of body weight) were significantly greater in the C3H mice than in the C57BL mice, respectively (not shown in table or graph form): liver 67.8 vs. 61.6 ($t = 3.2291$, 8 df, $p < 0.01$), left kidney 7.7 vs. 6.4 ($t = 4.2512$, 8 df, $p < 0.005$), and right kidney 8.3 vs. 6.9 ($t = 7.0072$, 8 df, $p < 0.0005$). However, the lung parenchyma 6.0 vs. 7.2 ($t = -5.7756$, 8 df, $p < 0.0005$) was significantly less in C3H mice as compared to C57BL mice.

Platelet Lifespans. The disappearance of labeled platelets over time for the mice in the first two experiments is illustrated in Figure 6. As shown, the percentage of labeled platelets decreased in a linear fashion. Lines depicted were determined by simple linear regression using all data points.

Platelet lifespans of C3H mice are represented in Figure 6A. For C3H mice, experiment 1 shows a platelet lifespan (x intercept) of 4.92 days, while experiment 2 shows a platelet lifespan of 4.77 days. Since the two experiments were not significantly different (comparison of intercepts: $p > 0.4$), the data were pooled and a platelet lifespan of 4.84 days was calculated.

Platelet lifespans of C57BL mice are represented in Figure 6B. For C57BL mice, experiment 1 shows a platelet lifespan of 5.75 days, while experiment 2 shows a platelet lifespan of 5.66 days. Since the two experiments were not significantly different (comparison of intercepts: $p > 0.4$), the data were pooled and the platelet lifespan for C57BL mice was calculated to be 5.70 days.

Pooled values for each strain of mice are shown in Figure 7. C3H mice had a platelet lifespan of 4.84 days, whereas, C57BL mice had a platelet lifespan of 5.70 days. The platelet lifespans of C3H and C57BL mice were significantly different ($p < 0.01$).

Effects of Splenectomy. Experiments were done on the splenectomized mice approximately 2 weeks after their splenectomy surgery. Platelet counts and packed cell volumes for splenectomized and intact mice are shown in Table 1. For C3H mice, splenectomy caused a slight increase in platelet counts ($p < 0.01$) and a decrease in packed cell volumes ($p < 0.0005$). Platelet counts in the splenectomized C57BL mice were elevated as compared to intact C57BL mice ($p < 0.005$) and packed cell volumes were decreased in the splenectomized C57BL mice ($p < 0.0005$) compared to the intact C57BL mice.

Platelet lifespans for C3H mice (intact and splenectomized) are represented in Figure 8A. For intact C3H mice, the data gave a platelet lifespan (x intercept) 4.85 days, while platelet lifespan values for splenectomized C3H mice were estimated to be 6.62 days. Platelet lifespans of intact and splenectomized C3H mice were significantly different (comparison of intercepts: $p < 0.01$).

Platelet lifespans for C57BL mice (intact and splenectomized) are represented in Figure 8B. Intact C57BL mice data gave a platelet lifespan (x intercept) of 6.66 days, while the data from splenectomized mice gave a lifespan of 6.62 days. Unlike C3H mice, platelet lifespans of intact and splenectomized mice were not significantly different in C57BL mice (comparison of intercepts: $p > 0.40$). Therefore, splenectomy caused an increase in the platelet lifespan of C3H mice ($p < 0.005$), but did not alter the platelet lifespan of C57BL mice ($p > 0.40$).

Hematology and Megakaryocyte Indices. For completeness, white blood cell counts, white blood cell differential counts, as well as, megakaryocyte size and numbers were investigated in 5 additional intact C3H and 5 intact C57BL mice. White blood cell counts and differential counts values are shown in Table 2. An unexpected finding was that C3H mice also had significantly higher neutrophil relative counts ($p < 0.005$) and significantly lower lymphocyte relative counts ($p < 0.005$) than the C57BL mice. However, after obtaining the absolute white blood cell count, C3H mice had only significantly higher neutrophil counts than did C57BL mice ($p < 0.005$)

Splenic megakaryocyte size and number for each strain of mice are shown in Figure 9. The average diameter of megakaryocytes (Fig. 9A) for the C3H mice was

significantly larger than the average megakaryocyte diameter for C57BL mice ($p < 0.005$). Splenic megakaryocyte numbers per unit volume for each strain of mice are shown in Figure 9B. The average megakaryocyte number for C3H mice was significantly greater than the average megakaryocyte number for C57BL mice ($p < 0.005$).

Histology. Sections of heart, lungs, kidneys, and spleen were examined via light microscopy. Although the spleens from the C3H mice were markedly larger than those of the C57BL mice, no major histological differences between the two strains of mice were detected in the sections of heart, lungs, liver and kidneys. The splenic cords within the red pulp of the C3H mouse spleen contained many megakaryocytes and the megakaryocytes appeared larger by visual observation than those from the C57BL mouse spleen. The larger megakaryocytes from the spleens of C3H mice had nuclei which appeared more mature than those seen in the spleens of the C57BL mice. The amount of erythropoiesis and myelopoiesis within the splenic cords of the red pulp was similar in both strains of mice. The white pulp appeared to be expanded in the spleens of the C3H mice. The C3H mice had larger lymphoid nodules and periarterial lymphatic sheaths. Often, the periarterial lymphatic sheaths and the lymphoid nodules coalesced into a smooth continuum in the C3H mice.

CHAPTER V.

DISCUSSION

The present study confirms previous work [1,4,7] and shows that C3H mice have lower platelet counts and lower packed cell volumes than C57BL mice. In addition, the present work demonstrates that C3H have higher serum testosterone levels, larger spleens, greater neutrophil counts, and larger and greater numbers of splenic megakaryocytes as compared to values from C57BL mice. Also, we show herein that intact C3H mice have a shorter platelet lifespan than values obtained for C57BL mice. When splenectomized, platelet lifespans of C3H mice were longer than values for intact C3H mice and approached the values observed for C57BL mice. Lengthening of platelet lifespan in C3H mice by splenectomy lends credence to the hypothesis that hypersplenism may be the cause of the thrombocytopenia in these mice, i.e., the data indicate that the spleens in C3H mice are destroying or sequestering platelets at a faster rate than found in C57BL mice. Therefore, this increased splenic activity may be the cause of the shortened platelet lifespans in C3H mice, leading to the lower platelet counts than have been found in other strains of mice.

As observed earlier [1,4,7], we found in the present study that C3H mice have significantly lower platelet counts than C57BL mice ($p < 0.0005$). The finding of decreased platelet counts in C3H mice compared to C57BL mice disagrees with one study that reported that the two strains of mice had similar platelet counts [10]. However, it should be mentioned that only 9 mice were used in this latter study and considering the variation and the methods that were used for determining platelet counts in these mice,

they did not use enough mice to justify a claim of similar or dissimilar values. Therefore, since our results are based upon several studies and the larger numbers of mice, it is believed that C3H mice do have lower platelet counts than C57BL mice. We hypothesize that the lower platelet counts seen in C3H mice are the result of hypersplenism in the C3H mouse, which would account for the increased platelet production, decreased platelet lifespans, and decreased platelet counts as compared to C57BL mice.

C3H mice also had significantly lower packed cell volumes than C57BL mice ($p < 0.0005$). A previous report [4] showed that the two strains of mice had similar packed cell volumes under normal conditions. However, other studies showed that C3H mice have a lower packed cell volume than BALB/c mice, which is another strain of mice with a 16N modal ploidy megakaryocyte [8,9]. One possible explanation for C3H mice having lower packed cell volumes is stem cell competition between the megakaryocytic and erythrocytic cell lines [76]. The stem-cell competition hypothesis is in agreement with the increased levels of testosterone found in the present study and also a previous finding [3] of increased percent ^{35}S incorporation into platelets of C3H mice as compared to C57BL mice. A shift in the production of the precursor cell to the megakaryocytic lineage would cause a decrease in red blood cell formation that was seen in C3H mice leading to a decreased PCV. Another hypothesis which is also supported by evidence in the present work is that the larger spleen may be sequestering not only platelets, but red blood cells resulting in the lower packed cell volumes that were seen in C3H mice as compared to C57BL mice. A final hypothesis would be that C3H mice may have a defective erythropoiesis system. However, there is no evidence to support this idea.

In addition to decreased platelet counts and lower PCV's, C3H mice have a greater percentage of neutrophils ($p<0.005$), but lower percentage of lymphocytes ($p<0.005$) than found in C57BL mice. However, absolute white blood cell counts show that C3H mice have only greater numbers of neutrophils than C57BL mice ($p<0.005$). Neutrophils are responsible for phagocytizing foreign material and lymphocytes are also involved in the identification of foreign proteins. Therefore, it seems possible that the neutrophils in the C3H mice may play a role in destroying platelets. Differential results from the Cell-Dyn were inconclusive since the machine appears to code C3H neutrophils as monocytes. An extensive literature search did not produce any reports showing that the white blood cell counts or differential counts were different in these two mouse strains.

Although it has been hypothesized that C57BL mice probably have higher testosterone levels because of their more aggressive behavior than other mouse strains [3], no literature exists to support this view. To determine if this hypothesis was true, we determined serum testosterone levels in both strains of mice. Although the results of the testosterone RIA were quite variable with several samples being outside the standard curve, C3H mice were shown to have significantly ($p<0.005$) higher testosterone levels than the C57BL mice. In support of this finding, it was shown earlier [3] that the percent ^{35}S incorporation into platelets was higher in C3H mice than C57BL mice, indicating increased platelet production in C3H mice. It was also reported that testosterone increases platelet production [8,53], since male mice of both strains have higher platelet counts, TCPC, and in most cases, higher percent ^{35}S incorporation than their female counterparts [3]. Therefore, increased testosterone levels in male mice causes increased

production of platelets and exogenous testosterone administered to castrated mice can restore their platelet counts to normal values [53]. C3H mice, with their elevated testosterone levels, also show an increase in platelet production as shown by their increased percent ^{35}S incorporation [9,52]. However, C3H mice have a decreased number of platelets in circulation as compared to C57BL mice. This observation can be explained by hypersplenism in C3H mice which would cause an increased number of platelets to be taken out of circulation and, therefore, cause the increased production and the mild thrombocytopenia.

When expressed as a percentage of body weight, C3H mice lung parenchyma was lighter than values for C57BL mice. However, C3H mice had larger livers and kidneys than the other strain of mice. Although an extensive literature search recovered no report of the tissue differences in these two strains, the larger kidneys reported in C3H mice could be the result of increased thrombopoietin production. It has been shown that the kidney produces thrombopoietin, but extrarenal sites of production may also exist [17]. The liver could possibly be another site of thrombopoietin production, since workers have shown that thrombopoietin is present *in vitro* in some liver cell media [77,78]. Increased thrombopoietin levels in C3H mice could explain the increased platelet production seen in C3H mice as compared to C57BL mice.

In agreement with the author's visual observations, C3H mice also had significantly larger spleens than found in C57BL mice. An exhaustive literature search did not provide the author with any information regarding normal spleen weights for the two strains of mice. It has been proposed that the size of the splenic pool of platelets is

determined solely by the size of the spleen [58,79]. Enlargement of the spleen is known to be associated with decreased platelet counts and has been termed 'hypersplenism' [58,80]. A hyperactive spleen can either sequester or destroy more platelets than normal. Therefore, an increase in the size of the C3H mouse's spleen (hypersplenism), and not suppression of platelet production, may be the cause of the decreased platelet counts.

C3H mouse spleens also had a darker appearance and upon histological investigation of spleen sections from the two strains of mice, it was determined that C3H mice have both increased size and number of megakaryocytes in the spleen as compared to C57BL mice ($p<0.005$). Although studies were performed utilizing splenic megakaryocytes of C3H mice [59], we are unable to find a report of direct comparison of splenic megakaryocytes of both C3H and C57BL mice. These observations may indicate a possible compensatory mechanism for the correction of the mild thrombocytopenia.

This study shows that biotinylation of murine platelets is a useful means of predicting platelet lifespans. Sulfo-NHS-Biotin randomly and stably labels the circulating platelets, making it easy to determine the percentage of labeled platelets left in circulation at any given time. In the present study, we determined that the platelet lifespan of male C3H mice (4.8 days) was significantly shorter ($p<0.01$) than C57BL mice (5.7 days). However, our results vary significantly from the data obtained by the use of ^{111}In in BALB/c mice where platelet lifespans of approximately 2.11 days were reported [63,64]. Although, our results agree with earlier work using ^{35}sulfur and ^{51}chromium that showed a platelet lifespan of about 4 days [62,65]. Recent work using biotinylation in mice to determine the age of certain sub-populations of platelets reported the platelet lifespans of

outbred Swiss-Webster mice to be 4.5 days [70]. Except for the work with ^{111}In, the small differences in our work and previous work could be due to the strain of mouse that was used or to the labeling techniques. Since flow cytometry allows every cell in a defined population to be individually analyzed, labeled platelets can be easily identified. Specific populations of radioactive cells cannot be analyzed in such a way. Also, unlike radiolabels, reutilization of Sulfo-NHS-Biotin is unlikely because the strongly reactive groups of the sulfo-N-hydrosuccinimide ester reacts with the primary amino groups in surface proteins of platelets. Even after a cleavage of the bond, e.g., by the action of biotinidase [81], reutilization of biotin could not occur because the biotinylation of the platelet proteins is based upon an irreversible split-off of the sulfo-NHS moiety, and mammalian cell systems have not been reported to form biochemically active forms of the vitamin biotin for cell labeling [82].

In preliminary experiments, identification of biotinylated platelets in whole blood was attempted, but was unsuccessful. Due to the small size of mouse platelets and the labeling of other blood constituents, it was required that platelets be washed free of plasma and other cell fragments prior to analysis. Once washed, the percentage of labeled platelets was easily measured. Because the FACScan is highly efficient, very small numbers of platelets are required for analysis. Although some decrease in labeling intensity does occur over time, the detection of labeled cells is not disturbed [70].

Because there is controversy over whether platelet lifespan models should utilize linear or exponential curves, an extensive literature search was done. The International Committee for the Standardization of Hematology has recommended the weighted mean,

multiple hit, and Dornhorst models for the estimation of platelet lifespans from data obtained using radiolabeling [83]. However, other mathematical models, including linear regression, have been suggested [84-87]. Because the results from our experiments followed a linear distribution, and the linear regression provided a good model fit, we have reported our results using this statistical model. A linear model supports the hypothesis that platelets are remove from the system based upon their age.

Biotin has been shown to be an appropriate label for platelets which can be applied *in vivo*. Using this technique, biotinylated platelets can be fluorescently tagged and analyzed using flow cytometry. This method provides a non-isotopic method for estimation of the mean platelet lifespan in mice. Using this method we have determined that the average platelet lifespan of intact C3H mice is 4.8 days, but that splenectomized C3H mice have an average platelet lifespan of 6.6 days. Whereas, intact C57BL mice have a calculated platelet lifespan of 5.7 days.

C3H mice have higher levels of testosterone and higher platelet production than C57BL mice, as shown by increased percent ^{35}S incorporation into platelets [9,52]. However, C3H mice have lower platelet counts and also lower total circulating platelet counts (TCPC) than the C57BL mice [1,4,7]. The lower platelet counts of C3H mice can be explained by the hypersplenism observed in the C3H mice. We believe that the more active spleen causes a shortened platelet lifespan.

In conclusion, we found that C3H mice, which have a modal ploidy megakaryocyte class of 32N, have increased platelet production, but lower platelet counts as compared to C57BL mice, which have 16N as their modal ploidy megakaryocyte class. In addition, we

discovered that C3H mice have several other differences from C57BL mice. First, C3H mice have higher testosterone levels, but lower PCV's than C57BL mice. This observation may be explained by the model of stem cell competition [76]. Obviously, in the C3H mouse, testosterone is acting to increase thrombocytopoiesis, and must therefore, not exhibit as great a stimulus on erythropoiesis. Compared to C57BL mice, C3H mice have larger spleens which contain more megakaryocytes with larger diameters. Intact C3H mice also have a shorter platelet lifespan than intact C57BL mice. However, splenectomy lengthens the C3H mouse platelet lifespan to values which are the same as C57BL mice. In conclusion, C3H mice have increased platelet production, but we believe that because of hypersplenism, C3H mice have a shortened platelet lifespan which results in their mild thrombocytopenia.

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APPENDIX

LIST OF ABBREVIATIONS

AO	Ammonium oxalate
ASA	Acetylsalicylic acid or aspirin
BW	Body weight
d	Profile diameter {d = 2[sq. root of (area/π)]}
D	Individual megakaryocyte diameter [D = 4(d/π)]
EDTA	Ethylenediaminetetraacedic acid
N_A	Number of section profiles per unit area
N_V	Number of spherical particles per unit volume [$N_V = N_A / (D = t)$]
PBS	Phosphate buffered saline
PCV	Packed cell volume
PRP	Platelet rich plasma
RAMPS	Rabbit anti-mouse platelet sera
RIA	Radioimmunoassay
SAB	Standard azide buffer
SAChE+	Small acetylcholinesterase positive cells
t	Thickness of the sample
TCPC	Total circulating platelet count (TCPC = platelet count/ml x blood volume in mls)
TCPM	Total circulating platelet mass (TCPM = TCPC x mean platelet volume)
TMM	Total megakaryocyte mass (TMM = average megakaryocyte volume x megakaryocytes/mm^3)
WBC	White blood cell

Table 1
Effect of Splenectomy on Platelet Counts and Packed Cell Volumes of C3H and C57BL Mice.

	C3H		C57BL	
	Intact	**Splenectomized**	**Intact**	**Splenectomized**
Platelet Counts (/μl x 10^{-6})	1.05 +/- 0.02 (62)*	1.14 +/- 0.03 (25)	1.17 +/- 0.02 (53)*	1.30 +/- 0.04 (29)
Packed Cell Volumes (%)	45.94 +/- 0.20 (51)*	43.59 +/- 0.29 (22)	48.33 +/- 0.32 (58)*	45.79 +/- 0.46(28)

Platelet Counts	C3H	Intact	vs. C57BL	Intact	$p < 0.0005$
	C3H	Splx	vs. C57BL	Splx	$p < 0.005$
	C3H	Intact	vs. C3H	Splx	$p < 0.01$
	C57BL	Intact	vs. C57BL	Splx	$p < 0.005$
Packed Cell Volumes	C3H	Intact	vs. C57BL	Intact	$p < 0.0005$
	C3H	Splx	vs. C57BL	Splx	$p < 0.0005$
	C3H	Intact	vs. C3H	Splx	$p < 0.0005$
	C57BL	Intact	vs. C57BL	Splx	$p < 0.0005$

* Restated here from Figures 2 and 3 for comparison purposes
** Values are Means +/- Standard Error (n)

Table 2
White Blood Cell (WBC) Counts and WBC Differential Counts of C3H and C57BL Mice.

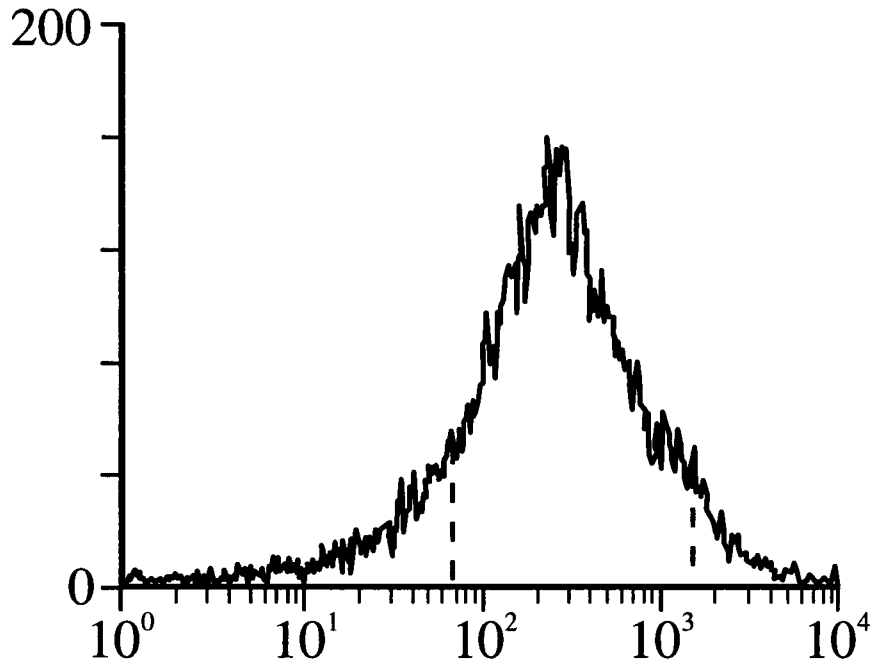
	C3H	C57BL	P-Values**
WBC Counts (K/μl)	1.264 +/- .08 (5)	1.381 +/- .20 (5)	ns
Neutrophils	.354 +/- .03 (5)	.157 +/- .04 (5)	< 0.005
Lymphocytes	.900 +/- .08 (5)	1.218 +/- .19 (5)	ns
Monocytes	.007 +/- .01 (5)	.002 +/- .00 (5)	ns
Eosinophils	.003 +/- .00 (5)	.004 +/- .00 (5)	ns
Basophils	.000 +/- .00 (5)	.000 +/- .00 (5)	ns
WBC Differential Count (%)			
Neutrophils	28.4 +/- 3.0 (5)	11.4 +/- 2.2 (5)	< 0.005
Lymphocytes	70.8 +/- 3.1 (5)	88.0 +/- 2.3 (5)	< 0.005
Monocytes	0.6 +/- 0.4 (5)	0.2 +/- 2.3 (5)	ns
Eosinophils	0.2 +/- 0.4 (5)	0.4 +/- 0.2 (5)	ns
Basophils	0.0 +/- 0.0 (5)	0.0 +/- 0.0 (5)	ns

*Values are Means +/- Standard Error (n)

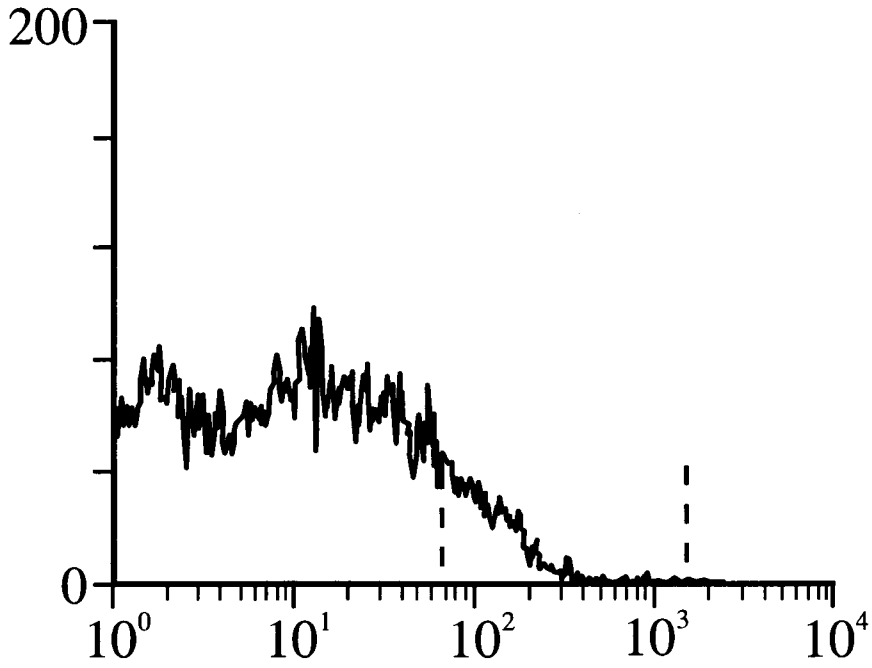
** P Values are statistical comparisons of data from C3H versus C57BL mice.

Figure 1.

Histograms of events versus log intensity showing fluorescence of platelet populations of C3H mice at both Days 1 and 4 after biotin treatment. The dotted vertical lines represent the gates which were set to determine the percentage of labeled platelets.



DAY 1



DAY 4

Figure 2.

Platelet counts of C3H and C57BL mice. C3H mice had lower platelet counts than C57BL mice ($t = -4.2456$, 113 df, * $p < 0.0005$). 62 C3H and 53 C57BL mice were used in these determinations. Vertical bars represent the standard error of the mean.

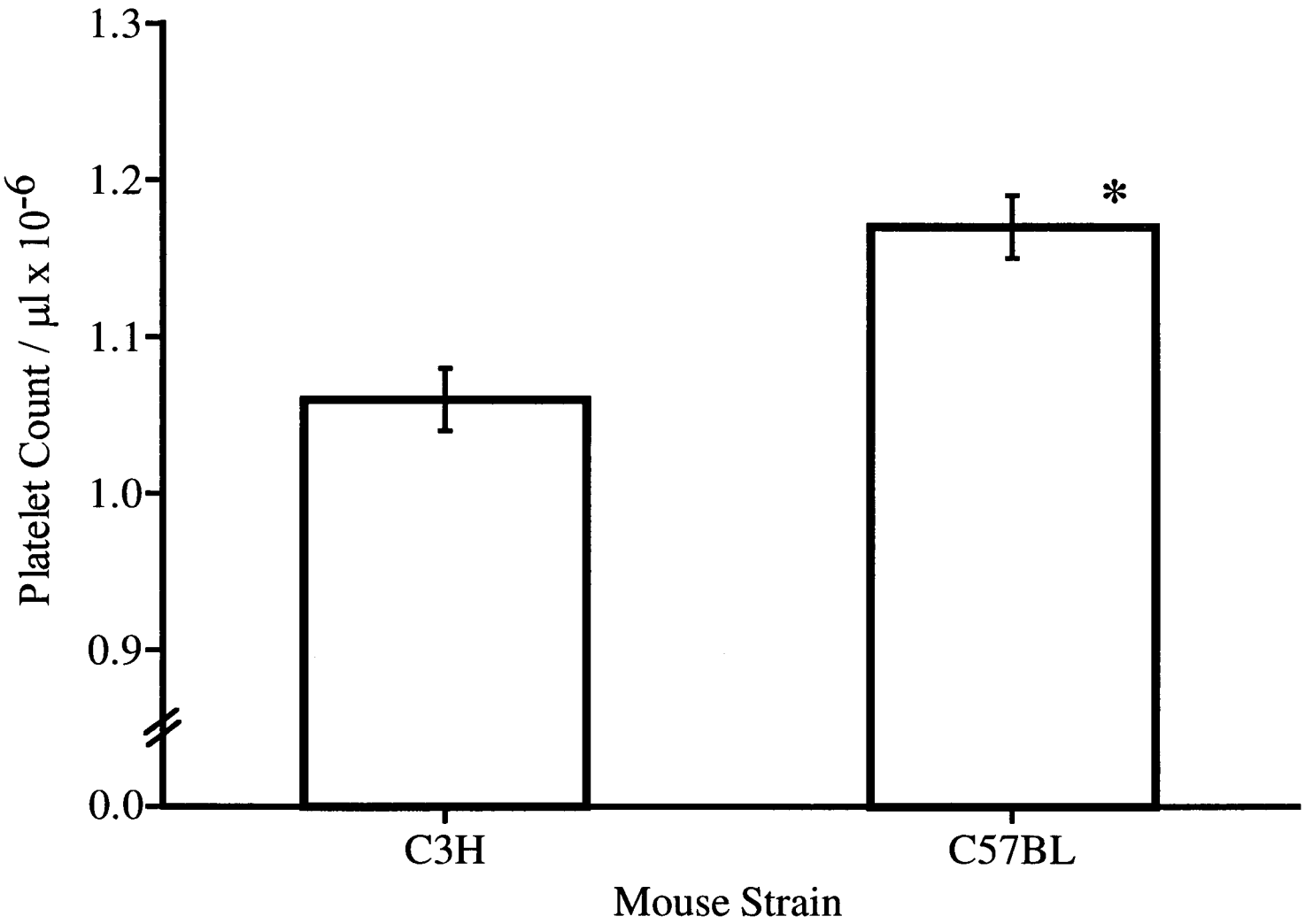

1.3
1.2
1.1
1.0
0.9
0.0
Platelet Count / µl x 10-6
*
C3H
C57BL
Mouse Strain

Figure 3.

Packed cell volumes for C3H and C57BL mice. C3H mice had significantly lower packed cell volumes ($t = -6.111$, 107 df, * $p < 0.0005$) than C57BL mice. These calculations were determined using 51 C3H mice and 58 C57BL mice. Vertical bars represent the standard error of the mean.

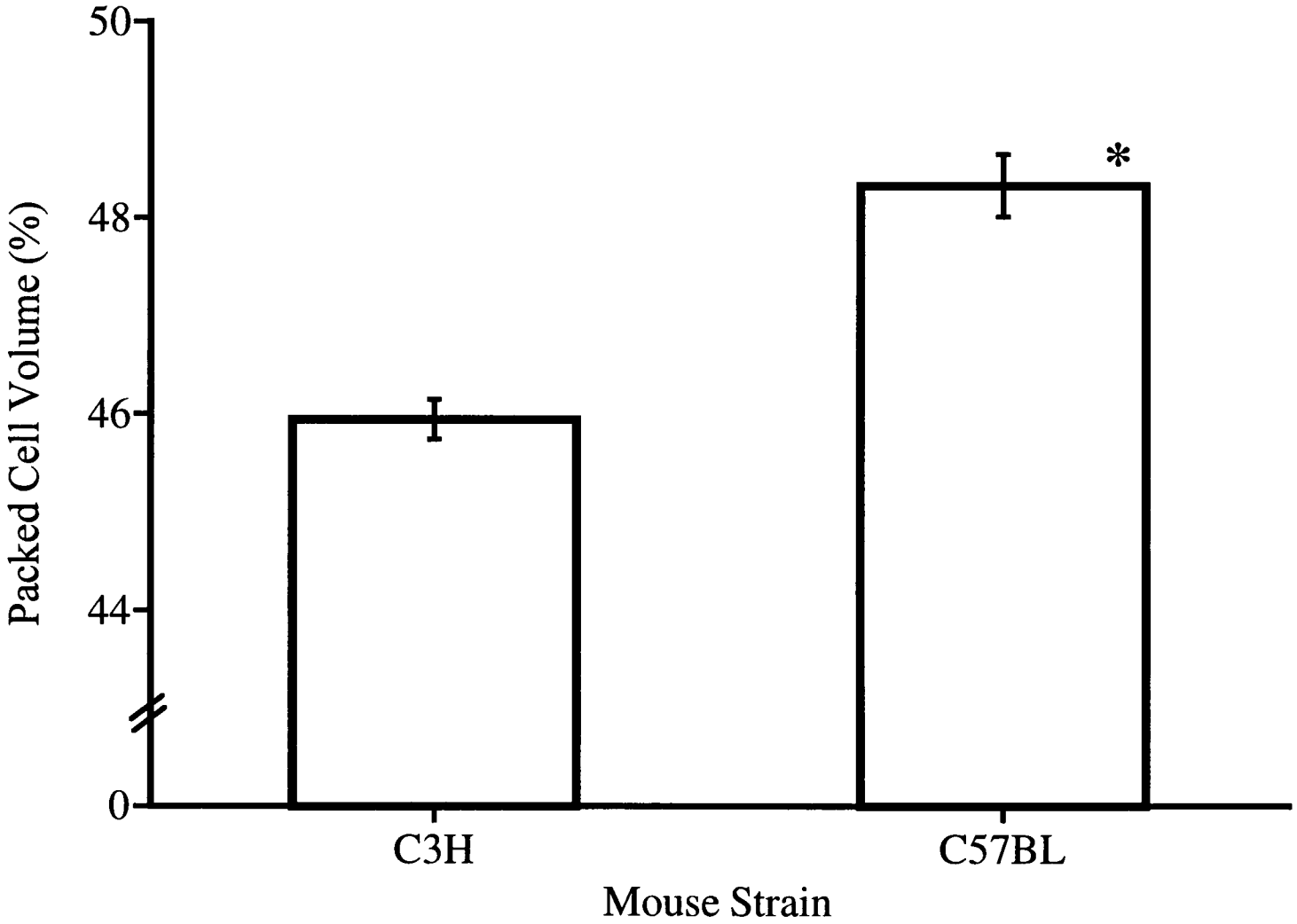

50
48
46
44
0
Packed Cell Volume (%)
*
C3H
C57BL
Mouse Strain

Figure 4.

Results of testosterone radioimmunoassay for C3H and C57BL mice. C3H mice had significantly higher testosterone levels than C57BL mice ($t = 3.1853$, 54 df, * $p < 0.005$). To obtain these results, serum from 30 intact male C3H and 26 intact male C57BL mice were assayed. Vertical bars represent the standard error of the mean.

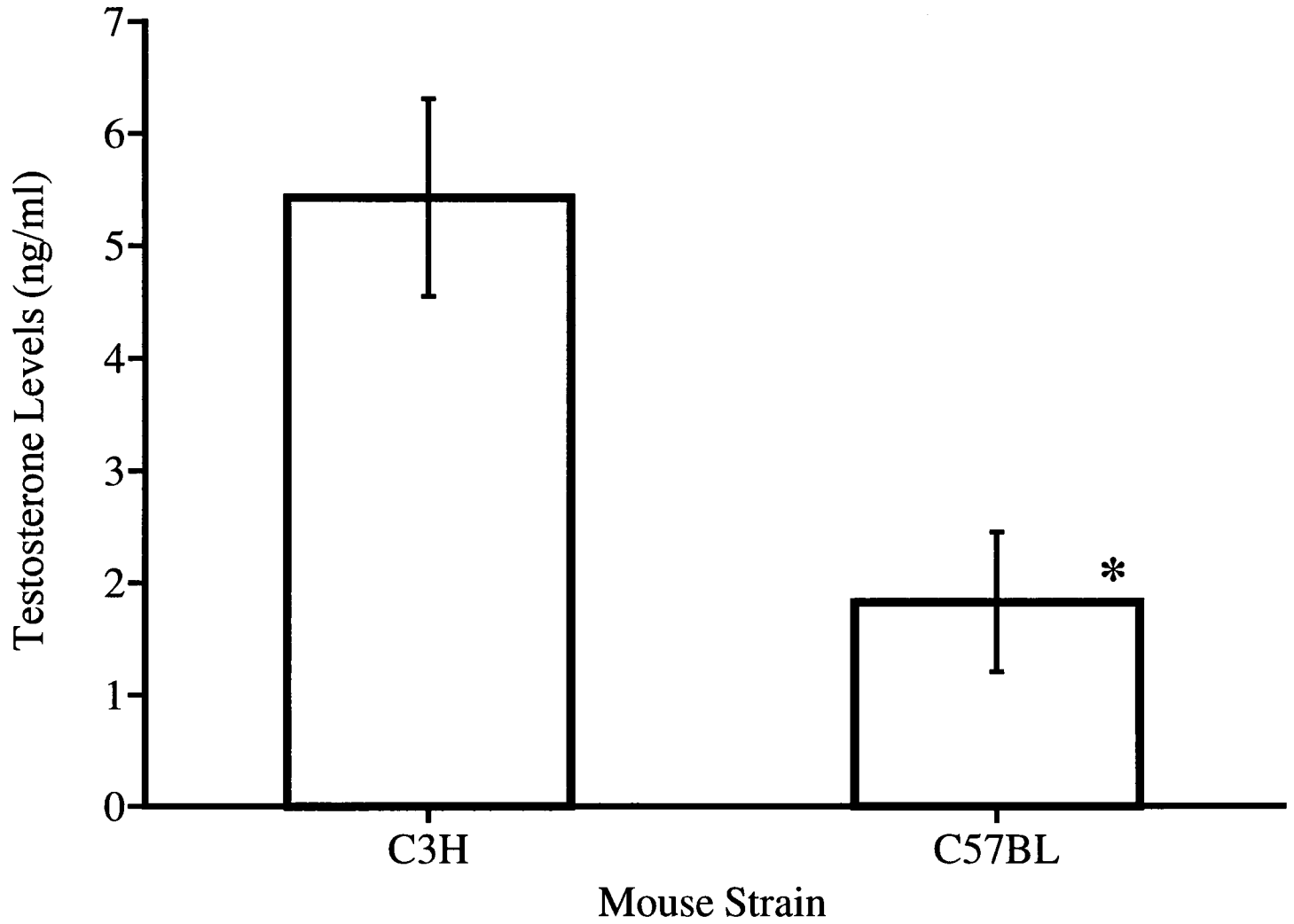

Testosterone Levels (ng/ml)
7
6
5
4
3
2
1
0
*
C3H
C57BL
Mouse Strain

Figure 5.

Spleen weights (mg / g of body weight) for C3H and C57BL mice. C3H mice (n = 60) had significantly larger spleens as a percentage of body weight than did C57BL mice (n = 52) ($t = -4.2456$, 113 df, * $p < 0.0005$). Vertical bars represent the standard error of the mean.

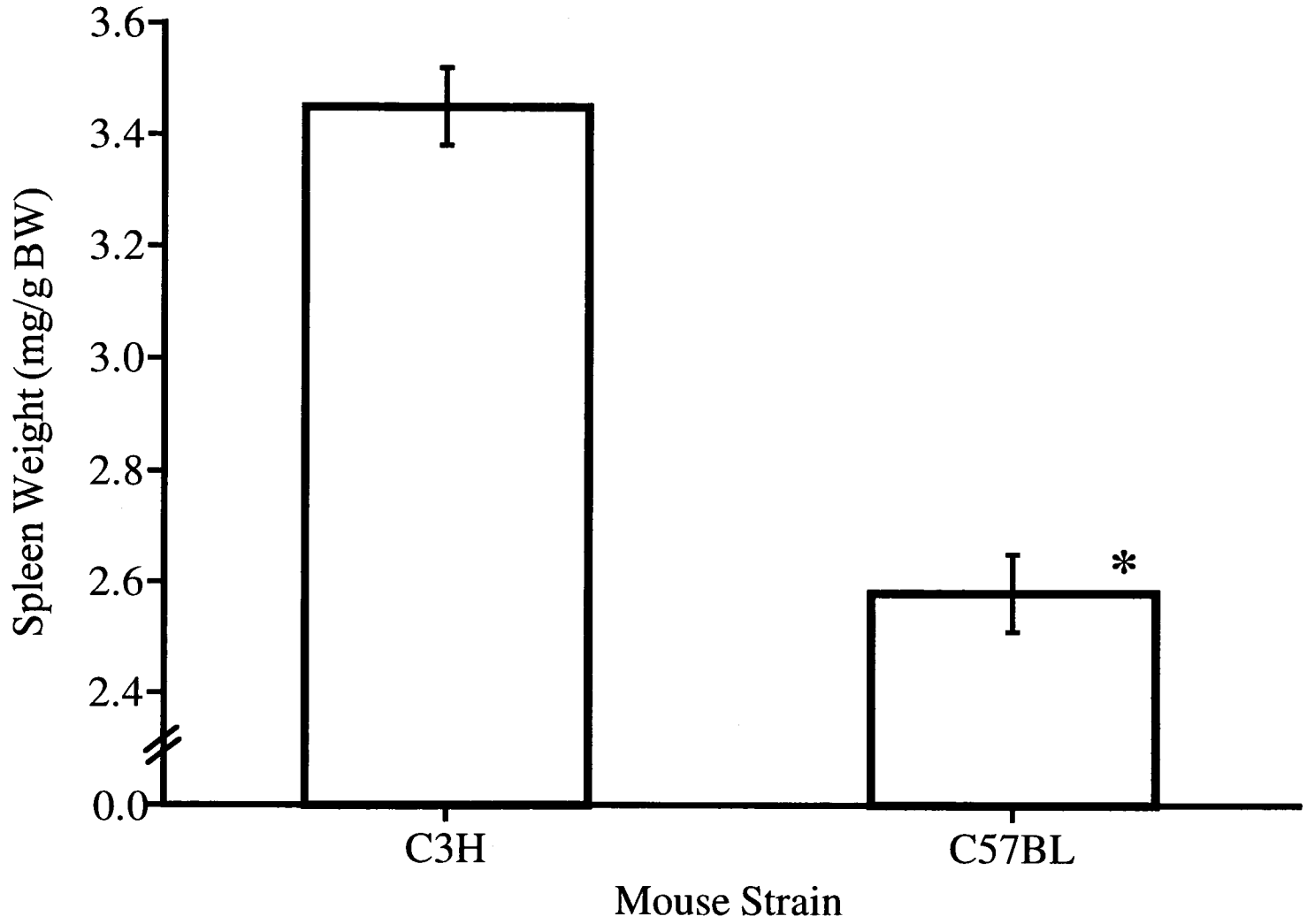

3.6
3.4
3.2
3.0
2.8
2.6
2.4
0.0
Spleen Weight (mg/g BW)
*
C3H
C57BL
Mouse Strain

Figure 6.

Platelet lifespans of C3H and C57BL mice.

A. Platelet lifespans of C3H mice. Data points represent the mean values for groups of two to four mice for each day. Experiment 1 shows a platelet lifespan (x intercept) of 4.92 days ($p = 0.0001$, $r^2 = 0.93$, $y = 100 - 20.31x$), while experiment 2 shows a platelet lifespan of 4.77 days ($p = 0.0001$, $r^2 = 0.93$, $y = 100 - 20.98x$). These two experiments were not significantly different (comparison of intercepts: $t = 0.02$, $df = 23$, $p > 0.4$). Vertical bars, although small, represent the standard error for each day.

B. Platelet lifespans of C57BL mice. Data points represent the mean values for groups of one to three mice for each day. Experiment 1 shows a platelet lifespan (x intercept) of 5.75 days ($p = 0.0001$, $r^2 = 0.96$, $y = 100 - 17.7x$), while experiment 2 shows a platelet lifespan of 5.66 days ($p = 0.0001$, $r^2 = 0.94$, $y = 100.02 - 17.67x$). These two experiments were not significantly different (comparison of intercepts: $t = 0.05$, $df = 18$, $p > 0.4$). Vertical bars, although small, represent the standard error for each day.

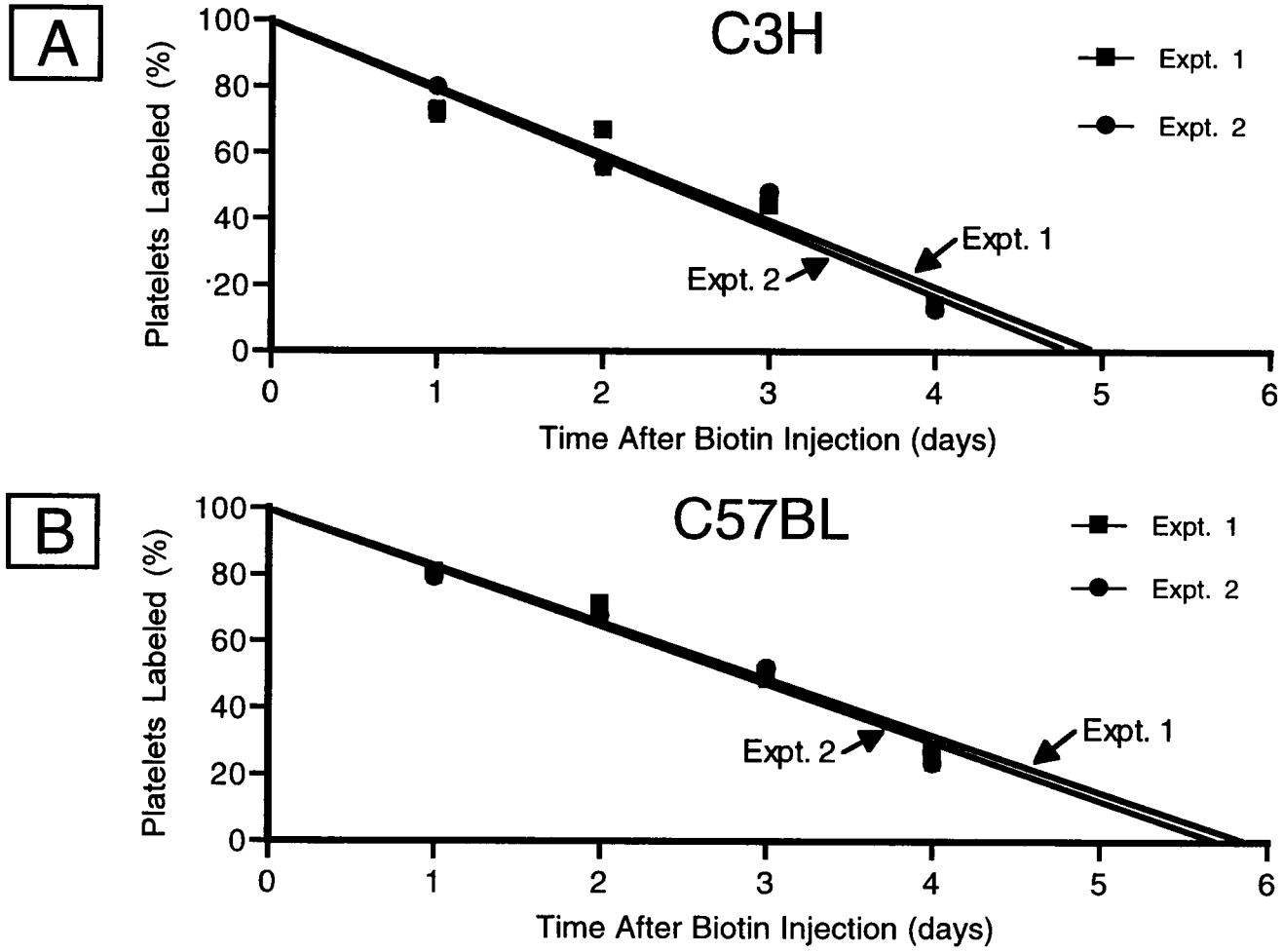

A
C3H
Platelets Labeled (%)
100
80
60
40
20
0
0
1
2
3
4
5
6
Time After Biotin Injection (days)
Expt. 1
Expt. 2
Expt. 1
Expt. 2
B
C57BL
Platelets Labeled (%)
100
80
60
40
20
0
0
1
2
3
4
5
6
Time After Biotin Injection (days)
Expt. 1
Expt. 2
Expt. 2
Expt. 1

Figure 7.

Pooled platelet lifespan values for each strain of mice. C3H mice have a calculated platelet lifespan of 4.84 days ($n = 25$, $p < 0.0001$, $r^2 = 0.93$, $y = 99.4 - 20.7x$), whereas C57BL have a platelet lifespan of 5.70 days ($n = 20$, $p = 0.0001$, $r^2 = 0.95$, $y = 99.96 - 17.7x$). C3H have a significantly shorter platelet lifespan than C57BL mice (comparison of t - intercepts: $t = -2.63$, $df = 41$, $p < 0.01$). Data points represent the means for groups of from four to six mice. Vertical bars represent the standard error for each day.

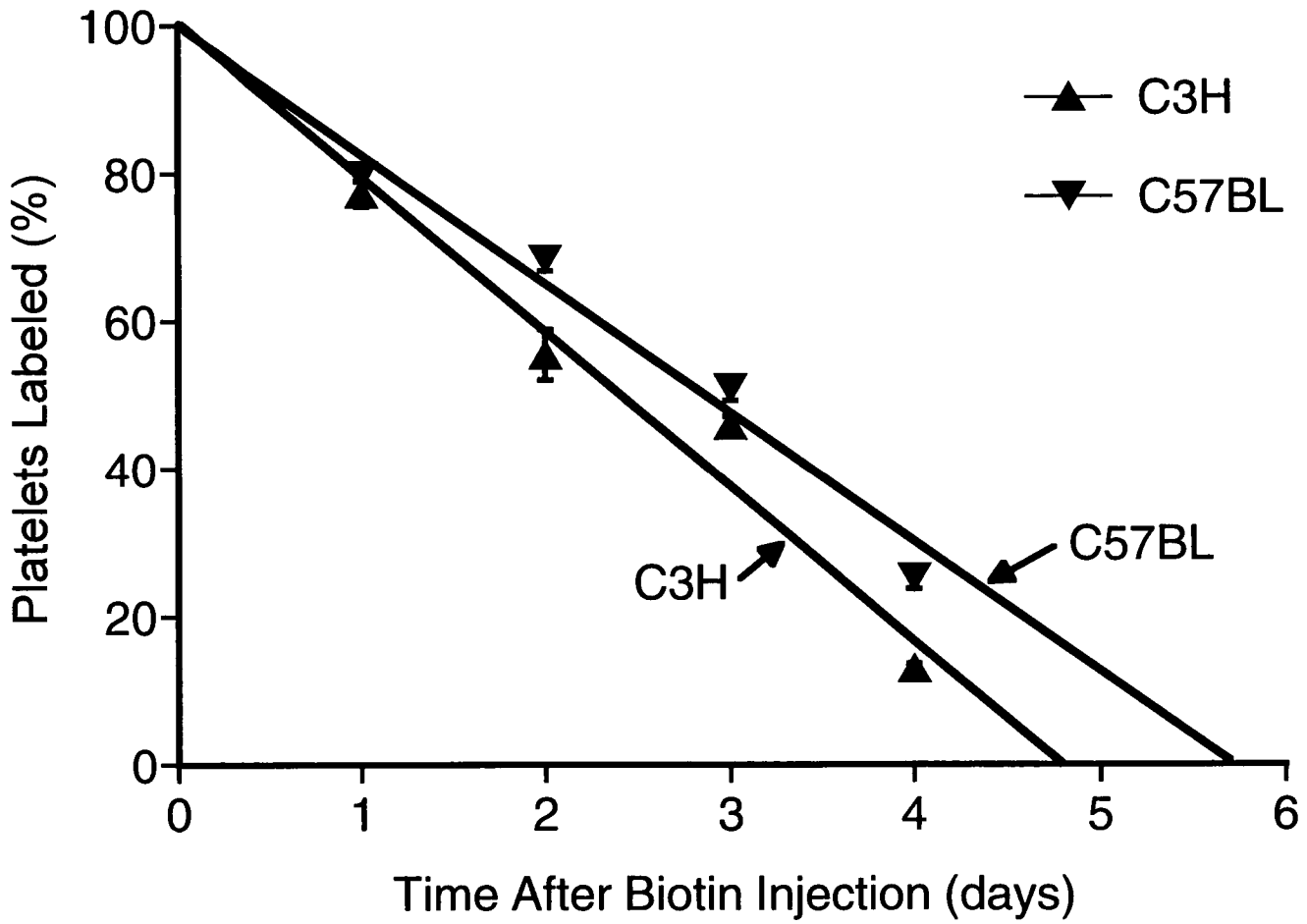

100
80
60
40
20
0
Platelets Labeled (%)
C3H
C57BL
C57BL
C3H
0
1
2
3
4
5
6
Time After Biotin Injection (days)

Figure 8.

Platelet lifespans for intact and splenectomized C3H and C57BL mice.

A. Platelet lifespans for intact and splenectomized C3H mice. For C3H mice, intact mice show a platelet lifespan (x intercept) of 4.85 days ($n = 13$, $p < 0.0001$, $r^2 = 0.89$, $y = 100 - 220.61x$), while splenectomized mice had a platelet lifespan of 6.62 days ($n = 8$, $p = 0.0021$, $r^2 = 0.82$, $y = 100 - 15.10x$). Splenectomy caused a significant increase in platelet lifespan (comparison of intercepts: $t = -2.56$, $df = 19$, $p < 0.01$). Data points represent means of groups of two to three mice. Vertical bars represent the standard error for each day.

B. Platelet lifespans for intact and splenectomized C57BL mice. For C57BL mice, intact mice show a platelet lifespan (x intercept) of 6.66 days ($n = 12$, $p = 0.0001$, $r^2 = 0.94$, $y = 100 - 15.01x$), while splenectomized C57BL mice had a platelet lifespan of 6.62 days ($n = 11$, $p = 0.0001$, $r^2 = 0.83$, $y = 100.0 - 15.10x$). Splenectomy did not change the platelet lifespan of C57BL mice (comparison of intercepts: $t = 0.066$, $df = 21$, $p > 0.40$). Data points represent means of groups of two to three mice. Vertical bars represent the standard error for each day.

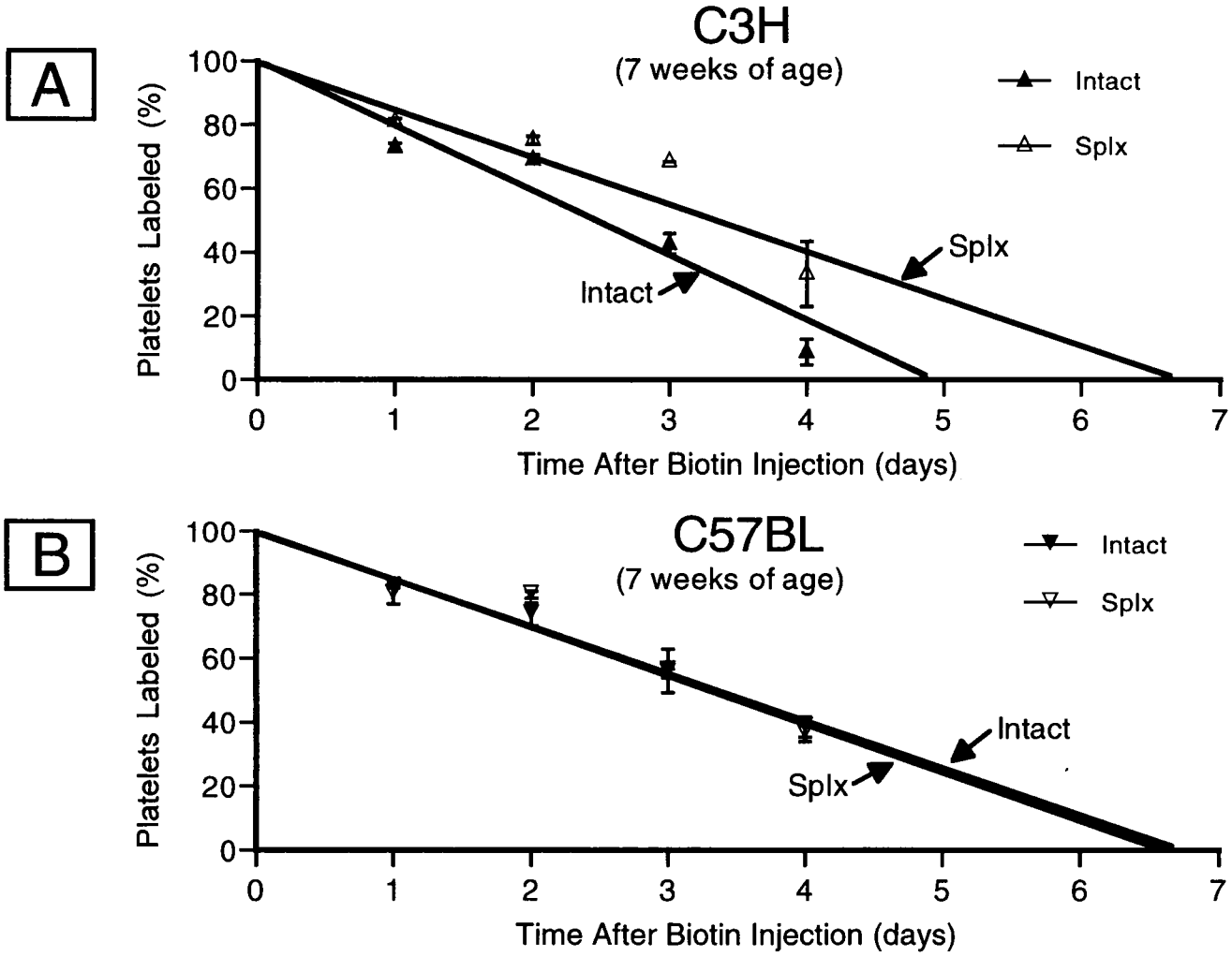
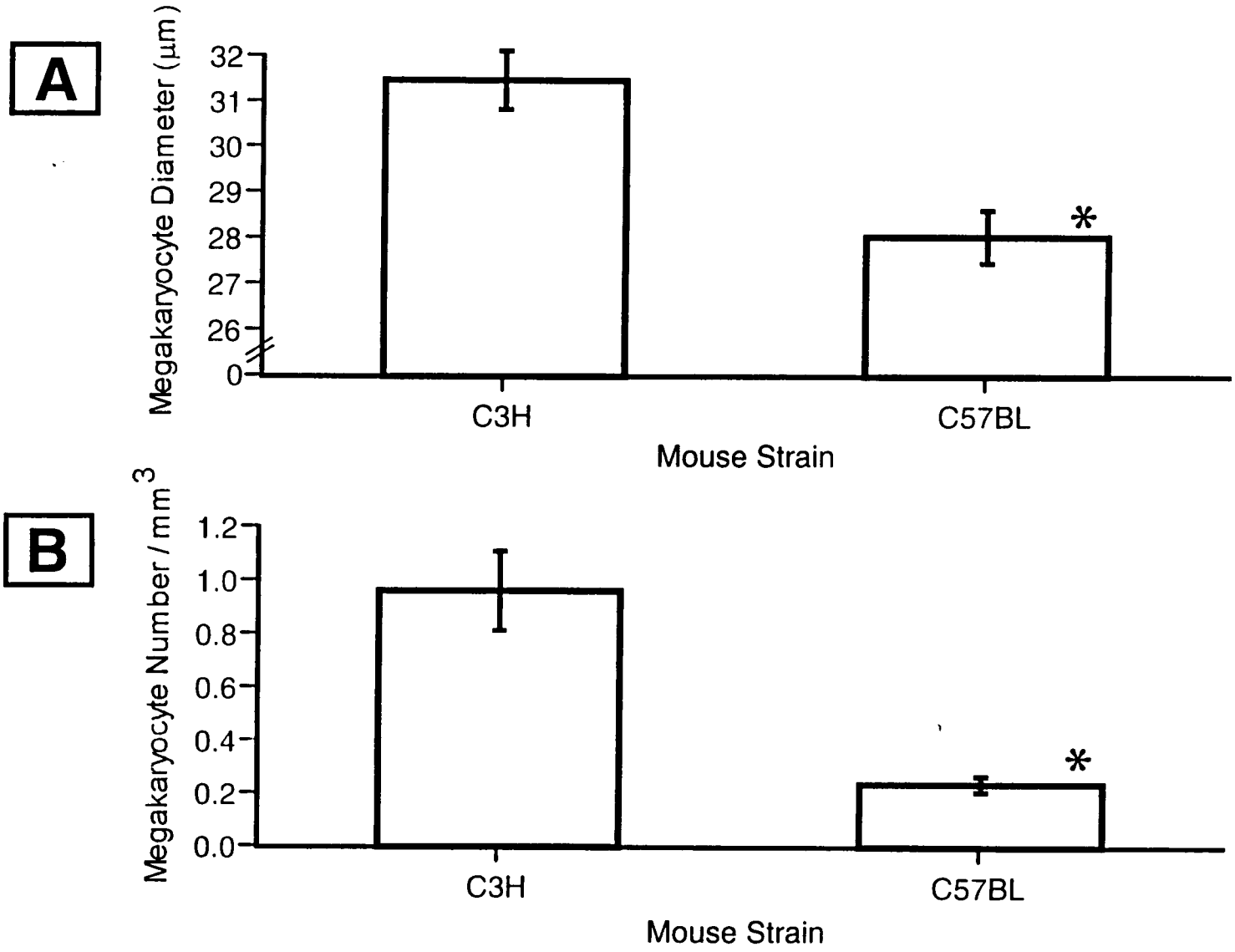

A
C3H
(7 weeks of age)
Intact
Splx
Splx
Intact
Platelets Labeled (%)
100
80
60
40
20
0
0
1
2
3
4
5
6
7
Time After Biotin Injection (days)
B
C57BL
(7 weeks of age)
Intact
Splx
Intact
Splx
Platelets Labeled (%)
100
80
60
40
20
0
0
1
2
3
4
5
6
7
Time After Biotin Injection (days)

Figure 9.

Splenic megakaryocyte size (A) and number (B) of C3H and C57BL mice. C3H mice (n = 5) have significantly greater megakaryocyte diameters ($t = 3.5226$, 8 df, * $p < 0.005$) and numbers ($t = 4.3426$, 8 df, * $p < 0.005$) than C57BL mice (n=5). Vertical bars represent the standard error of the mean.


A
Megakaryocyte Diameter (μm)
32
31
30
29
28
27
26
0
*
C3H
C57BL
Mouse Strain
B
Megakaryocyte Number / mm3
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
C3H
C57BL
Mouse Strain

VITA

Kendra Lynn Manning was born on October 8, 1973 to Don and Gina Manning. She attended McKenzie High School in McKenzie, Tennessee and was graduated Valedictorian in May 1991. In three and a half years, she received her Bachelor of Science degree in Agriculture with a major in Animal Science (December 1994) from The University of Tennessee, Knoxville. During her undergraduate career, she was an active member of Delta Gamma Sorority, Alpha Zeta Honorary Fraternity, Block and Bridle Club, Poultry Science Club, and the College of Agriculture Student/Faculty Council. In May 1996, she received a Master of Science degree in Comparative and Experimental Medicine from The University of Tennessee, Knoxville. While attending graduate school, she served as the Graduate Student Association representative for the College of Veterinary Medicine.