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QUANTITATION OF MEGAKARYOCYTE SIZE, NUMBER AND DISTRIBUTION DURING ALTERED MEGAKARYOCYTOPOIESIS IN MICE

A Thesis

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ABSTRACT

The objective of this study was to validate quantitative methods for determining megakaryocyte diameter and number in the bone marrow and spleen following stimulated-- rabbit antimouse platelet serum (RAMPS)-- and inhibited--isobaric hypoxia at 5% ambient oxygen for 14 days--megakaryocytopoiesis in mice. In addition, cell size and number were compared between bones, cell diameter was compared between smear and histological section preparations and two general quantitative methods for calculating megakaryocyte diameter and number were compared for precision and ease of use.

Following treatment, blood was collected for the measurement of common hematologic parameters. The mice were then sacrificed and the sternum, femur, humerus, tibia and spleen were collected and processed for light microscopic quantitation. Megakaryocyte diameter was calculated from frequency distributions of cell diameters after correction for optically lost profiles. The number of megakaryocytes per unit volume of bone marrow or spleen was then calculated from mean cell diameter and an estimate of the number of cell profiles per unit of section area after correcting for errors due to tissue section thickness. Both RAMPS and 14 days of continuous hypoxia produced increased mean megakaryocyte diameter suggesting stimulation of megakaryocyte maturation. However, megakaryocyte number was significantly increased after RAMPS and significantly decreased

after hypoxia, suggesting that RAMPS stimulates and hypoxia inhibits stem cell differentiation into megakaryocytes. The relative diameters of the megakaryocytes in the bone marrow were similar to the spleen, but the spleen had significantly lower numbers than in the bone marrow. Based on statistical estimates of precision, megakaryocytes in section preparations can be modeled as spherical for all treatments (hypoxia, RAMPS and control) with minimum error. Megakaryocytes flatten out on smear preparations, thus increasing their diameters. Also, fewer megakaryocytes were found using the smear compared to the section preparations. Under normal conditions, a 63% increase in diameter of the megakaryocytes was found in smear preparations compared to the section preparations. However, smear preparations showed constant and reproducible results suggesting that megakaryocyte diameters may be interconverted from smear preparations to section preparations by multipling the smear diameter by a factor of 0.63.

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1. INTRODUCTION

Quantifications of megakaryocyte diameter and number have proven useful in studying stimulated and inhibited megakaryocytopoiesis. Clinically, many diseases including acute leukemia, chronic myeloid leukemia and polycthemia vara leukemia are associated with an increase in medullary megakaryocyte diameter (Ebbe and Phalen, 1979) and number (Shreiner et al., 1980). Quantifications of megakaryocyte diameter and number may lead to earlier and more accurate diagnosis and prognosis of diseases associated with altered megakaryocytopoiesis.

Stereology is a family of mathematical methods based on geometric probability which estimates three-dimensional values of an entire structure from two-dimensional measurements on sections of the structure. The use of stereology avoids errors in the quantification of megakaryocyte size and number. In the past, many researchers have used biased parameter and sampling techniques in order to measure megakaryocytes. Such techniques are the reason why the literature contains many inconsistencies in the measurement of megakaryocyte size and number.

The major objective of this study was to analyze the validity of quantitative methods for determining megakaryocyte size and number during stimulated and inhibited megakaryocytopoiesis. Four specific aims within the major objective were established:

1. To compare between bones for differences in megakaryocyte size and number in bone marrow of different bones.
2. To compare smears versus histological sections for differences in megakaryocyte size.
3. To compare quantitation methods to see which offered the greatest precision.
4. To determine the lowest number of megakaryocyte profiles needed for statistical reliability in quantitation.

2. LITERATURE REVIEW

An historical perspective

Megakaryocytopoiesis may be defined as the differentiation, growth and maturation of megakaryocytes leading to the production of blood platelets. Megakaryocytes were first described by Robin as large cells with a diameter of 15-18 μm located in the medullary canal of bone (Robin, 1849). In 1890, William Howell named the large cell "megakaryocyte" because of its large nucleus (mega=large, karyo=nucleus, cytes=cells) (Howell, 1890). Howell thought the function of the megakaryocyte was associated with the formation of blood. Bizzozero was the first to recognize the polyploid nature of megakaryocytes by describing mitotic figures (Bizzozero and Sul Midollodelle Ossa, 1869). Polychrome staining showed similarities in the staining characteristics of megakaryocytes and platelets and was used to prove that platelets were made from megakaryocytes (Wright, 1906).

Megakaryocytes have been found in the bone marrow (Robin, 1849), circulatory system (Howell and Donohue, 1939), lung (Howell and Donohue, 1939) (Trowbridge et al.,1982), spleen and embryonic liver (Keleman, 1979). It was originally thought that the presence of megakaryocytes in the blood was a pathologic condition, until later studies showed a small number of megakaryocytes in the blood of normal animals (Minot, 1922). Both megakaryocytes and platelets

have been found in the capillaries of the lung. Howell and Donahue (1939) found megakaryocytes trapped in the lung and found more platelets leaving the lung than entering. It has been suggested that megakaryocytes produce platelets by physical fragmentation in the lung (Trowbridge et al., 1982). Both megakaryocytes and platelets in the lung seem to be a normal phenomenon. Also, megakaryocytes and platelets have been found in the mammalian spleen. In normal conditions, only a small number of megakaryocytes can be found in the spleen; however, up to 1/3 of the total platelet population can be found in the splenic cords at any one time (Erslev and Gabuzda, 1979). Both the liver and spleen serve to destroy megakaryocyte nuclei and platelets through phagocytosis by macrophages (Erslev and Gabuzda, 1979).

A model for megakaryocytopoiesis

All hematopoietic cells, including megakaryocytes, arise from a crude multipotential stem cell of mesenchymal origin, called the colony-forming-unit spleen (CFU-S). Till and McCulloch (1961) were among the first to show evidence for the existence of a multipotential stem cell. They transplanted normal adult bone marrow cells into irradiated recipients and found nodules in the spleen containing erythroblasts, granuloblasts, and megakaryoblasts (Till and McCulloch, 1961).

A series of events must happen in order for an uncommitted stem cell to reach the stage of a mature megakaryocyte: commitment, cellular proliferation, nuclear proliferation (endomitosis), and cytoplasmic maturation. In rodents, the committed cell changes morphologically into a small acetylcholinesterase postive (AChE+) cell, then into a megakaryoblast, a basophilic megakaryocyte, and finally into a mature megakaryocyte (Levine, 1981).

After specific stimulation, CFU-S cells differentiate into one of three kinds of committed stem cells: erythrocytes (CFU-E), granulocytes/ monocytes (CFU-GM), and megakaryocytes (CFU-M) as shown in Figure A1*. For the megakaryocyte this specific stimulation is thought to be the humoral agent, megakaryocyte colony stimulating factor (Meg-CSF) (Hoffman et al., 1981). Other CSF's are found for the other cell lines CFU-E and CFU-GM (Erslev and Gabuzda, 1979) (Junqueir and Carneriro, 1983). Meg-CSF stimulates CFU-S cells to become CFU-M. Further differentiation of CFU-M is stimulated by thrombopoietin (TSF), which mainly affects maturation and growth. In rodents, after stimulation by TSF, CFU-M gives rise to a sequence of more differentiated cells, including the small acetylcholinesterase positive cell (SAChE+ cell) stage one,

*All figures and tables are in the appendixes.

followed by stage two, stage three, and stage four megakaryocytes as shown in Figure A2 (Levine, 1981).

The first cell in the developmental sequence in rodents is the SAChE+ cell, or stage one megakaryocyte. Structurally, the SAChE+ cell is a small cell with a diameter of < 13 μm, a nonsegmented nucleus, and agranular cytoplasm. Also, one or two mitotic divisions have occurred in the SAChE+ cell stage one megakaryocyte (Levine, 1981). SAChE+ cells stain for the enzyme acetylcholinesterase (AChE). AChE can be used to identify any cell in the rodent megakaryocyte developmental sequence. The enzyme acetylcholinesterase can be found in both eosinophils and megakaryocytes; however, due to differences in cytoplasmic components one can differentiate SAChE+ cells from eosinophils. Eosinophils have granular cytoplasm, as opposed to SAChE+ cells which have agranular cytoplasm (Levine, 1981).

The next cell in the developmental sequence is the stage two megakaryocyte, or megakaryoblast. Structurally, the megakaryoblast is an immature megakaryocyte with a lobed kidney-shaped nucleus, numerous nucleoli and an intensely basophilic, agranular cytoplasm. The size of the megakaryoblast is 13-20 μm in diameter with a high nucleus-to-cytoplasm ratio. The basophilic cytoplasm indicates a high concentration of free ribosomes (Levine, 1981). Endomitotic

generations could have occurred through this stage in the developmental sequence (Mazur, 1986).

The next cell in the developmental sequence is the stage three, or mature basophilic megakaryocyte. Structurally, this cell is larger in diameter than the megakaryoblast and contains a multilobed nucleus and a moderate number of azurophilic granules in the cytoplasm. Also, the cytoplasm is basophilic due to free ribosomes. Compared to stage one, the nucleus-to-cytoplasm ratio is reduced. A maximum of five endomitotic generations could have occurred through this stage in the developmental sequence (Mazur, 1986).

The next cell in the developmental sequence is the stage four mature, or platelet-producing megakaryocyte, which is no longer undergoing endomitosis. Structurally, this cell is characterized by a condensed multilobed nucleus and azurophilic granules in the cytoplasm and has the lowest nucleus-to-cytoplasm ratio of the three previous cells in the series. The stage four cytoplasm contains more azurophilic granules than either stage one or stage two (Levine, 1981).

When a megakaryocyte reaches the later stages of maturation, it releases its platelets into the circulatory system. At the light microscopic level, platelets are irregular, non-nucleated masses containing two zones: the peripheral zone (hyalomere) and the central zone (granulomere). The hyalomere stains a pale blue color, and the granulomere stains a purple color (Erslev and Gabuzda, 1979).

At the electron microscopic level, processes are noticed budding from the hyalomere zone. With the Wrights blood stain, the hyalomere zone contains electron-dense granules (azurophilic granules), mitochondria, glycogen granules, vacuoles and marginal bundles of microtubules. The microtubules are thought to maintain the shape of the platelets. The granulomere contains glycogen and electron-dense granules, mitochondria and vacuoles. Some of these electron-dense granules contain adenosine diphosphate (ADP) or serotonin, or are lysosomes released during the process of aggregation. Glycoproteins and glycosaminoglycans form a 15-20 nm thick glycocalyx on the surface of the platelet (Erslev and Gabuzda, 1979).

Megakaryocytes producing platelets can be divided into three major ploidy classes: approximately 1/6 of the platelets are from the 8N ploidy megakaryocytes; 2/3 of the platelets are from the 16N ploidy megakaryocytes; and 1/6 of the platelets are from the 32N ploidy megakaryocytes. Each megakaryocyte ploidy class has its own platelet characteristics (Queissner et al., 1971) (Penington, 1979). The 8N ploidy megakaryocytes produce platelets that are large and dense. These large, dense platelets have increased numbers of granules and decreased membrane thickness. Small, light platelets with an increased membrane thickness and a decreased granule content are produced by 32N and 64N megakaryocytes. The 16N megakaryocytes produce platelets with intermediate structural

characteristics. It also seems that ploidy level has an effect on the function of the platelets, since larger platelets have been shown to have more hemostatic ability than smaller platelets (Penington, 1979).

The principle function of platelets is to seal vascular damage by forming a hemostatic plug at the site of injury. Platelets adhere to the subendothelial collagen at the site of injury which triggers the release of ADP, serotonin, prostaglandins and thromboxanes. Aggregation of platelets occurs in two waves. Both waves depend on the amount of ADP present. In the first wave, a small amount of ADP changes the shape of the platelet from a disk to a sphere. In the second wave, larger amounts of ADP cause an irreversible aggregation of the platelets. Serotonin released by the platelets acts as a vasoconstrictor, contracting the vascular smooth muscle. More platelets aggregate until the vascular damage is sealed. Both the platelets and the vascular endothelial cells release thromboplastin, which converts prothrombin to thrombin. Then platelet factor three is released from platelets to augment thrombin conversion. Thrombin converts fibrinogen to fibrin, which helps trap other platelets. After the vascular injury is sealed, platelets release thrombostenin which retracts the clot (Erslev and Gabuzda, 1979).

Thrombopoiesis

Platelet production and release

Early researchers thought that platelets were precursors of erythrocytes or breakdown products of the blood (Donne, 1842). The production of platelets from megakaryocytes in the bone marrow was first shown by Wright in 1906.

Bone marrow was first suggested to be the site of platelet production. The mechanism proposed was that of continuous budding (Wright, 1906). The theory of continuous budding was supported by the discovery of naked nuclei in the bone marrow and cinemographic studies made by inserting a glass window in the femur of a rabbit (Kinostia and Ohno, 1961). Although Wright thought that platelets were produced by continuous budding from the edges of the megakaryocytes, continuous budding has been questioned by some (Paulus et al., 1979) (Radley and Scurfield, 1980) (Trowbridge et al., 1982). It is now also known that megakaryocytes spread on glass, which could have produced artifacts in Kinostia's study (Trowbridge et al., 1982) .

Recent ultrastructural data suggest another mechanism of platelet release. A single release mechanism of platelets from megakaryocytes was proposed by Paulus in 1979, based on the demarcation membrane system (DMS) observed previously in megakaryocyte cytoplasm (Paulus et al., 1979). Demarcation

membranes were thought to divide platelet territories within the megakaryocyte cytoplasm. Megakaryocytes have been shown to develop cytoplasmic processes which extend into sinusoids. These processes contain DMS, which serve as a reserve for platelet membrane. Thus, it was thought that the processes undergo attenuation and constriction, followed by fragmentation, to liberate platelets (Paulus et al., 1979).

Another theory for platelet release is the filamentous process theory (Radley and Scurfield, 1980). Megakaryocytes have been observed to undergo significant morphological change before platelet release (Wright, 1906). Studies using scanning electron microscopy have shown megakaryocytes developing filamentous processes (Scurfield and Radley, 1981) (Becker and DeDruyn, 1976). These filamentous processes are thought to be putative platelets joined end to end which undergo constriction to liberate individual platelets. Longitudinally oriented microtubules were found in these processes, specifically at the constriction areas. A cell in mitotic division has a similar microtubule bridge as was noted in the processes. Thus, it has been suggested that a common mechanism occurs between the dividing cell and the platelet release from megakaryocytes.

Physical fragmentation of the megakaryocyte cytoplasm in the pulmonary circulation has been proposed as a mechanism of platelet release (Trowbridge et al., 1982). As the blood vessels in the lung get smaller and smaller, fragmentation of megakaryocytes occurs.

This theory was tested using a rat heart/lung preparation. In this system, perfused fluid flowed from the pulmonary artery to the pulmonary veins to the left atrium (Slater et al., 1983). Fluid, containing particles, was collected in the left atrium and analyzed. After megakaryocytes entered the circulation of the lung, the particle count rose. Ultrastructural analysis showed these particles to be platelets. Also, naked nuclei were seen coming out of the lung, which also suggested physical fragmentation (Trowbridge et al., 1982).

Roles of bone marrow and spleen in the production of platelets

Two organs, the bone marrow and the spleen, were found to be important in platelet production. To understand the relative roles of the bone marrow and the spleen in the production of platelets, ^{89}Sr injection and splenectomy were used (Layendecker and McDonald, 1982). An injection of ^{89}Sr caused bone marrow aplasia for at least 30 days. Platelet counts, platelet size and incorporation of ^{35}S into platelets were used as a measure of thrombocytopoiesis. In splenectomized mice, platelet production, platelet size and count were within normal values. After the removal of bone marrow from normal mice, the count decreased while the platelet size increased. An increase in size was thought to compensate for the low number of platelets. Also, the ^{35}S incorporation increased. It was concluded that under normal conditions, the spleen contributed little to the

production of platelets, however, the spleen produced platelets when bone marrow production ceased (Layendecker and McDonald, 1982).

In another study, 5-Fluoruracil (5-FU) injection was used to determine the roles of the spleen and the bone marrow in the production of platelets. One injection of 5-FU stimulated the bone marrow by increasing megakaryocyte diameter seven days post-injection. By 17 days post-injection, 5-FU caused thrombocytosis, an increase in the splenic number of megakaryocytes and an decrease in megakaryocyte diameter, while the bone marrow produced an increased granulocyte production (Radley and Scurfield, 1980). An autoregulation mechanism has been proposed to explain an increase in diameter and an decrease in the number of megakaryocytes under stress, i.e., radiation and hypoxia (Edde and Scurfield, 1980). Also, it has been proposed that the spleen microenvironment was enhanced by increased megakaryocyte sensitivity to TSF (Radley and Scurfield, 1980).

Intrinsic and humoral factors controlling megakaryocytopoiesis and thrombopoiesis

Megakaryocyte Colony Stimulating Factor (Meg-CSF)

Chemical nature of Meg-CSF. Meg-CSF has been purified and partially characterized from urine (Enomoto et al., 1980), serum

(Hoffman et al.,1985) and plasma (Kimura et al., 1984). Purified Meg-CSF from human plasma showed a glycoprotein with a molecular weight of 46,000 (Hoffman et al., 1981). Meg-CSF has been suggested to be a glycoprotein because of its affinity for Coomassie Blue, PAS (periodic acid shiff), and wheat germ lectin; all of which reveal carbohydrate moieties. When Meg-CSF carbohydrate oligosaccharides were cleaved by TFMSA (trifluoromethane sulfonate), Meg-CSF lost the ability to form colonies in vitro (Hoffman et al.,1985). The level of Meg-CSF varied with different diseases, which may indicate a physiological role (Hoffman et al., 1981).

Biological effects of Meg-CSF. The model of megakaryocytopoiesis has benefitted not only from the technical advances in histology, but from the technical advances in molecular biology as well. Meg-CSF worked mainly on early cells in the megakaryocytes series; however, thrombopoietin (TSF) worked mainly on the later development (i.e., postmitotic period) (Williams et al., 1979). It has been shown that erythropoietin (Epo) may be a megakaryocyte colony stimulating factor (Meg-CSF). Previously, human Epo from urine stimulated megakaryocyte colonies one out of four times in in vitro experiments (Williams et al., 1984). However, more recently, in vitro experiments showed recombinant Epo (r-Epo) stimulating megakaryocyte colonies twice as much as human Epo

(Dukes et al., 1986). Also, the evidence showed that r-Epo stimulated an increase in megakaryocyte precursor cells in in vivo experiments. It has been suggested that human Epo contained impurities (other plasma and urinary proteins), which may have inhibited the full effect of Epo to stimulate megakaryocytes in vitro and in vivo (Dukes et al., 1986). It has been shown that IL-3 (interleukin-3) may be a Meg-CSF. IL-3 has been found in WEH-I3 cell condition media stimulating megakaryocyte colonies. IL-3 and Meg-CSF have similar molecular weights. Both Meg-CSF and IL-3 stimulated activities were inhibited by a rabbit anti-Meg -CSF serum (Mizoguchi et al., 1986).

Thrombopoietin (TSF)

Chemical nature of thrombopoietin. Studies of the chemical nature of thrombopoietin (TSF) have shown conflicting data (McDonald, 1973a). In the past, thrombopoietin was thought to be a glycoprotein (Linman and Pierre, 1964), an alpha-globulin (Schulman et al., 1965), a beta-globulin (Rak, 1963), or an albumin molecule (Steinberg et al., 1959). Currently, the TSF molecule is considered to be a protein. McDonald has shown a two-fold increase in alpha-globulins using a TSF-rich fraction compared to whole serum (McDonald, 1973a). This increase seems to indicate that TSF may be an alpha-globulin glycoprotein (McDonald, 1973a). In fact, most

investigators accept TSF to be a relativetly heat stable glycoprotein (Schulman et al., 1965) (Chat et al., 1965).

The collection of TSF from both human embryonic kidney (HEK) cell culture medium and plasma of thrombocytopenic animals has been attempted. Purification of TSF from HEK cell culture medium showed a glycoprotein with an isoelectric pH of 4.7, a molecular weight of 32,000, and a specific activity of 11,000 units per milligram (McDonald et al., 1986) (McDonald et al, 1987). However, the extraction of TSF from the plasma of thrombocytopenic animals was less successful due to the ineffectiveness of purifying methods (McDonald, 1973a) (Evatt et al, 1979) (Dassin et al., 1983) (Hill and Levin, 1986).

TSF functions as a hormone. In order for a molecule like TSF to be a hormone, four criteria must be met:

1) A similar effect (e.g. thrombocytopenia) after transfer of plasma from one animal to another,
2) A decrease in production when the endproduct (platelets) is added,
3) The presence of a dose response, and
4) The ability to elicit antibody production.

All of the these characteristics have been shown to occur for the TSF molecule (McDonald et al., 1980b) (Kalmaz and McDonald, 1982).

Site of TSF production and stimulation of production. The releasing mechanism of TSF into the circulatory system has been difficult to study. The site of release, non-specific factors, and releasing factors had been the main problems in studying the mechanism of TSF release into the circulatory system (Cooper, 1970). The spleen, kidney, and the platelets themselves have been proposed by researchers as the sites of TSF release (Abildgaard and Simone, 1967) (Cooper, 1970).

In the past, studies of the site of TSF production have shown conflicting results. Some investigators have suggested that the spleen (Cooney et al., 1965) or the kidney (McDonald and Clift, 1976) or the liver (Siemensma et al., 1975) were important in the production of TSF. The evidence seems to indicate that the kidney is at least one site for TSF production. McDonald showed evidence both in vitro and in vivo for TSF production in the kidney (McDonald, 1976). Using human embryonic kidney cells in culture, TSF production was found and tested for biological activity in thrombocytopenic mice. In thrombocytopenic mice, produced by platelet-specific antisera, the removal of the kidney stopped TSF production in vivo (McDonald, 1976) (Krizsa et al., 1977) .

The removal of the liver from mice led both to decreased ploidy in megakaryocytes and to thrombocytopenia (Siemensma et al., 1975); however, liver cells in culture did not produce TSF (Ogle et al., 1978) (Zucali et al., 1977). Nutritional problems associated with

the removal of the liver may account for both the decreased ploidy and the thrombocytopenia. The spleen has been proposed to be a site of TSF production (Cooney et al., 1965). However, in rodents, the removal of the spleen produced no decrease in platelet count (Krizsa et al., 1971).

Assays for TSF. Many of the problems associated with older indirect assays for TSF were overcome by present-day technical advances in direct assays. Non-specific and releasing factors effected the degree of sensitivity of the earlier indirect assays for TSF. However, platelet counts (McDonald, 1981) (McDonald et al., 1977), platelet sizes (McDonald, 1980a) (Levin et al., 1982) and $\%^{35}S$ (McDonald, 1981) (McDonald, 1973c) or $\%^{75}Se$ (Evatt and Levin, 1969) incorporation into platelets have all been used successfully as indirect assays for TSF. The most sensitive of the three was the $\%^{35}S$ incorporation.

In platelet counting, plasma from thrombocytopenic mice was injected into recipient mice. Three days after injection of TSF plasma, mice were sacrificed and blood was removed for platelet counting (See Appendix C). This method in itself caused a problem, since in mice, an increase in the platelet count occurred when a foreign material was injected into a normal recipient (O'dell et al., 1964).

In the platelet sizing, mice were sacrificied and blood was removed two days after an injection of TSF. Blood was centrifuged at 160 X G for 4.5 minutes to obtain the platelet button. Platelet sizes were determined by a particle counter interfaced with a computer. Platelet sizing required special instruments such as a computerized particle sizer which are not available in most laboratories (McDonald et al., 1980a).

In the third indirect assay, the $\%^{35}S$ incorporation into platelets of mice was measured following injection with RAMPS or TSF from HEK cell culture medium. One injection of RAMPS or TSF from HEK were given on day one and two injection of RAMPS or TSF from HEK were given on day five and six. 30 μCi ^{35}S was given on day seven and mice were sacrificed by cervical dislocation on day eight. Blood was then drawn and centrifuged at 800 X G for 15 minutes to form the platelet button. The platelet button was washed in 1% ammonium oxalate, centrifuged at 800 X G, and washed again in 1% ammonium oxalate and saline. The percent of radioisotope incorporated into the platelets was then measured. The % ^{35}S and % ^{75}SeM incorporation of isotopes have been shown to a be sensitive assay; however, cost, time, non-specific factors (i.e., infection, stress, exercise, and surgery), releasing factors (plasma proteins and phospholipids) and the many proposed releasing sites made this assay less useful (McDonald, 1973a) (Kalmaz and McDonald, 1982).

Since direct assays were not affected by non-specific or releasing factors, the hemagglutination-inhibition assay for TSF seemed to have overcome the problems of the assays used previously (McDonald, 1973b). Thrombocytopenic sheep were used in the production of TSF-rich sera by injecting them with rabbit antisheep platelet serum (ASPS). After extraction, TSF-rich sera was partially purified using column chromatography. Thrombocythemic mice injected with TSF-rich sera were used to test for biological activity. For the production of antithrombopoietin (Anti-TSF), methylated rabbit serum albumin chemically bound to TSF was injected into rabbits to immunize them. Antibodies not specific to TSF were removed by microabsorption of antisera with normal sheep sera. Hemagglutination (HA) occurred when anti-TSF antibodies and sensitized indicator RBC combined to make complexes. Anti-TSF antibodies plus sensitized indicator cells made large complexes (3+ or more) when mixed. When the sites on anti-TSF were blocked, a negative reaction occurred (no complexes). Thus, the amount of TSF can be measured by how much inhibition occurred (McDonald, 1973b).

In the past, it was noticed that RAMPS caused an increase in % SAChE+ cells (Jackson, 1973), while injection of platelets decreased the % SAChE+ cells (Long et al., 1981). It was suggested that early precursor cells might have been a sensitive indicator of the changes in thrombocytopoiesis (Kalmaz and McDonald, 1985). Kalmaz compared the increase in % SAChE+ cells versus control after

injecting RAMPS or TSF from kidney cell culture or plasma from thrombocytopenic animals. Ten hours after injection, blood was collected from the retroobitial sinus for platelet counting. Femoral marrow was removed and mixed with plasma expander followed by AChE staining for three hours at room temperature. Marrow was then postfixed, counterstained and the number of SAChE+ cells were counted. A dose-dependent response was found. An increase in doses of RAMPS or TSF caused an increase in the % of SAChE+ cells. This direct assay has been shown to be more sensitive and less expensive than the ^{35}S incorporation into platelets in detecting low amounts of TSF (Kalmaz and McDonald, 1985).

Additionally, it has been suggested that megakaryocyte diameter may be a sensitive indicator of the changes in throbocytopoiesis (Cullen and McDonald, 1986). In a preliminary study, a positive dose-response relationship was found between increasing doses of TSF and increasing mean megakaryocyte diameters (Kalmaz and McDonald, 1985).

Likewise, fetal mouse liver cells (FMLC) have been shown to be more sensitive than adult bone marrow cells in producing AChE+ cells (Kalmaz and McDonald, 1985). An FMLC assay has not as yet been developed.

Biological effects of thrombopoietin. TSF was shown to have an effect on cytoplasmic maturation (Williams et al., 1984) (Kalmaz and

McDonald, 1985), size (McDonald and Kalmaz, 1983) (Cullen and McDonald, 1986), and endomitosis (O'dell et al., 1969) (McDonald, 1981) of megakaryocytes. TSF affected both early and later cells in the sequence of development. The main effect of TSF seemed to be on the more mature cell, rather than on the earlier cells in the series. Using a serum-free assay, TSF has been shown to increase megakaryocyte colony formation (Hoffman et al., 1986). In in vitro studies, TSF has been shown to increase numbers of the megakaryocyte colonies (early cells) in the bone marrow (Kalmaz and McDonald, 1981) (Freedman et al., 1981) and liver cells (Kalmaz and McDonald, 1985) in mice. TSF played a smaller role in the development of early cells than it played in later development, including endomitosis and cytoplasmic maturation (Kalmaz and McDonald, 1985). In vivo ,TSF caused an increase in the isotopic uptake into platelets (McDonald, 1981) (McDonald et al., 1985), an increase in platelet counts (McDonald, 1981) (O'dell, 1974), and an increase in platelet size (Weintrab and Karpatin, 1974). In another study, TSF enhanced the transit of SACHE+ cells in a wave-like response to develop into immature and then mature megakaryocytes (Raha et al., 1985). Mice were injected with TSF and sacrificed at 10.5 and 18 hours after injection. Then their bone marrow was processed and stained with ACHE. Both SACHE+ cells and immature megakaryocytes were significantly increased at 10.5 hours; however, only immature megakaryocytes were significantly increased at 18

hours. Thus, these results support the theory that the main effect of TSF is on the maturation of megakaryocytes.

The endomitotic index was stimulated by injecting TSF from HEK culture cells, or by injecting thrombocytopenic plasma from thrombocytopenic animals to another. In one study, after injecting a mouse with TSF from HEK culture cells or plasma, the megakaryocyte numbers increased significantly (O'dell et al., 1979). Likewise, when a C_3H mouse was injected with RAMPS, which caused thrombocytopenia, there was a significant increase in the endomitotic index compared to saline controls (O'dell et al., 1979). The ploidy (DNA level) significantly increased four to five days after the onset of thrombocytopenia. Normally, the ploidy class of megakaryocytes had a mode of 16N colonies in vitro, but because of thrombocytopenia the ploidy class shifted to between 32N and 64N. Also in in vivo studies, the increase in ploidy class could be seen in bone marrow after thrombocytopenia. DNA quantity influenced the cytoplasmic volume of megakaryocytes along with the number of platelets produced per megakaryocyte. Studies have shown the higher the ploidy number, the greater the platelet volume. Thus, injection of partially purified plasma TSF increased both ploidy values and mean platelet volume (Levin et al., 1982)

TSF is a major controlling factor stimulating megakaryocytes in a thrombocytopenic animal (Harker, 1968). TSF stimulated megakaryocytopoiesis by increasing committed diploid precursor

cells and then by transforming the cells into mature megakaryocytes (Kalmaz and McDonald, 1981). Sources of TSF from both exogenous human embryonic kidney cell culture (HEK) (McDonald and Kalmaz, 1983) and endogenous plasma (Harker, 1968) caused an increase in both number and diameter of megakaryocytes. An increase in number indicated an increase in the population growth; an increase in diameter indicated an increase in maturation.

In the past, the use of peripheral platelet counts and platelet size as an indicator of thrombocytopoiesis produced conflicting data. In recent studies, it has been shown that platelet counts only increased significantly using high doses of plasma from thrombocytopenic mice or TSF from HEK cell culture (McDonald, 1980a) . An increase in the $\%^{35}S$ has been shown for both plasma from thrombocytopenic mice and TSF from HEK cell culture (McDonald, 1980a). Also, the use of $\%^{35}S$ incorporation into platelets has been shown to be more sensitive than either the platelet counting or sizing assay. This assay used low doses of TSF to produce increases in the $\%\ ^{35}S$ incorporation. Thus, both sources of TSF (plasma and HEK) have been shown to increase the peripheral platelet counts, platelet size and the incorporation of $\%^{35}S$ into platelets (McDonald, 1980a).

Platelets

The demand for platelets seems to be the principal modulator of megakaryocytopoiesis. The need for more platelets seems to affect the size of the platelets that will be produced (Martin et al., 1983). In an effort to supply that need, megakaryocytes shed larger platelets. An animal increases its platelet mass, size and platelet number, within one to two days after stimulation. An increase in isotope uptake was shown within six hours by Martin el at. in 1983 using a bioassay.

Changes in the circulating platelet number and size have an effect on megakaryocytes in the bone marrow. A reduction in the numbers and sizes of megakaryocytes was shown following hypertransfusion of rats with platelets (Harker, 1974). This reduction was measured using ^{35}Se-selenomethionine. Thus, platelet hypertransfusion decreased the number of mature megakaryocytes (Harker, 1974).

The removal of platelets from the circulatory system caused an increase of TSF into plasma (Abildgaard and Simone, 1967) from the kidney (McDonald and Clift, 1976). It has been shown that TSF increases SAChE+ precursor cell numbers (Raha et al., 1985), endomitosis (Kalmaz and McDonald, 1985), and diameter and number of megakaryocytes (Harker, 1968) (McDonald and Kalmaz, 1983).

Effects of hypoxia and Antiplatelet Serum on megakaryocytopoiesis and thrombocytopoiesis

Effects of hypoxia

Hypoxia has been shown to inhibit megakaryocytopoiesis (Jackson and Edwards, 1977) (McDonald and Kalmaz, 1983) (Cullen and McDonald, 1986). Several studies have suggested autoregulation of megakaryocyte size and number (Ebbe and Phalen, 1979) (McDonald et al., 1985) (Burstein et al., 1980) (Paulus et al., 1981). In conditions like hypoxia, an increase in size may have compensated for the low numbers of megakaryocytes in mouse bone marrow. A relationship has been found between low numbers of megakaryocytes and megakaryocytic macrocytosis in vivo (Ebbe and Phalen, 1979).

Other studies have shown that with an increase in mature megakaryocytes, the number of megakaryocyte progenitors decreased. In rodents, megakaryocytes make acetylcholinesterase. Blocking or decreasing acetycholinesterase production increases megakaryocytic colonies in vitro (Burstein et al., 1980). Neostigmine, an acetycholinesterase inhibitor, increased megakaryocyte colonies by 62% and increased the number of cells in DNA synthesis by 45% in vitro (Burstein et al., 1980).

Hypoxia has a biphasic effect on the platelet count in mice. The effect of short term hypoxia (one to three days) was to increase the platelet count (Cooper and Cooper, 1972) (Jackson and Edwards,

1977); however, long term hypoxia (fourteen days) decreased the platelet count (Birks et al., 1975) (Langdon and McDonald, 1977) (McDonald, 1978). In short term hypoxia, platelet numbers increased in mice as shown by the uptake of $\%^{35}S$ and $\%^{75}Se$ isotopes (McDonald, 1978). Humoral agents like TSF were known to affect platelet numbers, but it took at least three days for TSF to be made in mice. It seems that the results of short term hypoxia on platelet production might have been due to the stress of the hypoxic condition or dehydration (Lail et al., 1970). In long term hypoxia, the platelet numbers decreased in mice as shown by the uptake of $\%^{35}S$ and $\%^{75}Se$ isotopes. Either an increase in blood volume (Lail et al., 1970) or splenomegaly (Penny et al., 1966) might have accounted for a decrease in platelet counts in hypoxic mice. An increase in blood volume was noticed, but it was not enough to reduce the platelet count significantly (Langdon and McDonald, 1977). A decrease in the platelet count was also noticed when hypoxia caused splenomegaly, which traps platelets in an enlarged spleen. When the spleen of an hypoxic mouse was removed, a greater reduction of platelet production was found than in splenctomized mice breathing ambient air. However, when one compared splenectomized-hypoxic mice to normal hypoxic mice, the reduction of platelets was not as great in splenectomized-hypoxic mice. Normal hypoxic mice had a higher platelet count. Thus, splenomegaly could not have accounted for all of the effect of hypoxia in the mice. It was estimated that a

reduction in the platelet count due to splenomegaly and spleen trapping of platelets accounted for half the reduction of the platelet population (Langdon and McDonald, 1977).

Additional questions to be answered were:

1) Could hypoxia directly interrupt thrombopoiesis?
2) Could hypoxia inhibit platelet production by stem cell competition?

In one experiment, Balb/c mice were used, which had a genetic defect in their erythropoietin production (McDonald and Clift, 1976). If hypoxia directly interrupted thrombopoiesis, then the Balb/c mice should have had a significantly reduced platelet count (thrombocytopenia). In a hypoxic condition, Balb/c mice had a significantly higher platelet count than C_3H mice. Thus, hypoxia could not be directly interrupting thrombopoiesis. The stem-cell theory was tested in experiments using RBC-transfused mice (McDonald, 1978). When red blood cells were transfused into the mice, the level of erythropoietin was lowered. In both experiments, lower levels of erythropoietin seemed to be associated with normal platelet counts, thus supporting the stem-cell competition theory between megakaryocyte and erythrocyte cell lines. Balb/c mice produced little or no erythropoietin while their platelet production remained normal. However, C_3H mice, with normal erythropoietin production in the hypoxia condition, showed increased erythropoietin levels and decreased platelet counts. Thus, an increase in

erythropoietin levels caused an increase in RBC production at the expense of platelet production.

Effects of antiplatelet serum

RAMPS has been shown to stimulate megakaryocytopoiesis, increasing both the number and size of megakaryocytes (McDonald and Kalmaz, 1983) in bone marrow. Using normal rats injected with TSF-rich serum from thrombocytopenic rats, O'dell showed an increase in the number of megakaryocytes in bone marrow (O'dell et al., 1964). Later, O'dell showed this same increase in thrombocytopenic rats after four daily injections of RAMPS (O'dell et al., 1969). A significant increase in megakaryocyte number could be seen as early as 18 hours after one injection into a normal rat (Jackson, 1973). However, the effect of RAMPS may not be felt uniformly throughout tissues associated with hematopoietic functioning (Enomoto et al., 1980).

In mice, the mechanism of RAMPS-induced thrombocytopenia is poorly understood. It is not known whether RAMPS acts directly or indirectly on platelets. To study platelet movement in the body, platelets were labeled with ^{35}S. RAMPS caused platelets to be aggregated (McDonald and Clift, 1976). Platelet aggregation had a biphasic wave effect. In the first wave response, platelet aggregation was followed by an reversible shape change of platelets. In the second wave response, irreversible platelet aggregation was

followed by a release of large amounts of ADP from the platelets. This releasing effect caused the platelets to lyse. Three hours after RAMPS injection, most of the platelets with ^{35}S-MPS (mucopolysaccharide) label were removed from the circulatory system and found in the kidney (McDonald and Clift, 1976)

Injection of RAMPS leads to severe thrombocytopenia in two to three days followed by rebound thrombocytosis in both normal and RBC transfused mice (McDonald, 1978). Injection of RAMPS caused a biphasic response in platelet size (McDonald, 1978). Compared to normal mice, platelet size was larger during the severe thrombocytopenia than during the rebound thrombocytosis. Therefore, platelets may be larger to compensate for the low number. An increase in % ^{35}S was found after RAMPS injection within three days; however, platelet production returned to normal after seven days.

Significance of measuring megakaryocyte size and number

Quantification of megakaryocyte size and number may prove a useful tool in diagnosing diseases such as acute leukemia, chronic myeloid leukemia and polycythemia vara (Ebbe and Phalen, 1979). An independent relationship can be found between DNA content and the size of megakaryocytes. An increase in DNA content usually follows an increase in the megakaryocyte size. Because of this relationship, megakaryocyte maturity has been estimated by

measuring the diameter or mean size. In one study, megakaryocyte ploidy was used to measure cell maturation (Levine et al., 1982). As the megakaryocytes matured, they increased in size. At each stage of maturation, the mean diameter increased with ploidy. When the ploidy doubled, the mean volume doubled in each of the four stages of development. Maturation stage and ploidy level independently influenced megakaryocyte size as shown using a two-way analysis of variance with log transformed data. Maturation and polyploidization were found to be linked. Low ploidy was associated with immaturity; however, high ploidy was associated with maturation. The growth of the megakaryocyte population has been estimated by counting megakaryocyte number (Levine et al., 1982).

Past methods for measuring megakaryocyte size and number

The idea of quantification of megakaryocyte size and number is not a new idea. In the past, researchers commonly used fixed (Levine et al., 1982) and unfixed (Ebbe et al., 1973) whole cells, squash (O'dell et al., 1970) and smear (Ebbe et al., 1981) (Levine, 1981) preparations to quantify megakaryocyte size and number. Recently, however, most reseachers have used histological sections of bone marrow to quantify megakaryocyte number and size.

Measurements of major and minor axes have been used to calculate the section profile diameter as an estimate of megakaryocyte size (Jackson and Edwards, 1977). Other methods

which have been used less frequently to measure megakaryocyte size include circle fitting (Hilliard and Cahn, 1961), planimetry (Ebbe et al., 1973) and cut out and weigh methods (O'dell et al., 1970). The number of cells per high-power microscopic field (HPF) has been commonly used to estimate megakaryocyte numbers (O'dell et al., 1969) (Krizsa et al., 1977).

Significant errors may be caused by cell shape and optically lost profiles when using section profile diameter as a measure of megakaryocyte maturity. Also, significant errors may be caused by section thickness when using the number of cell profiles as a measure of the megakaryocyte population growth. All of these errors can be compensated (Weibel, 1979).

Stereology

Stereology is a family of mathematical methods based on geometric probability which is used to estimate three-dimensional values of a structure from measurements made on two-dimensional sections of that structure. Stereology contains methods which will compensate for many of the most common errors in the quantification of megakaryocyte size and number. In the past, many researchers have used biased parameters and sampling techniques.

These biases may be the reasons why inconsistencies in the measurements of the number and size of megakaryocytes are common in the literature.

Geometric probability

Stereologic methods in biology are both geometrical and statistical in nature (Weibel, 1979). Stereologic methods also deal with probabilities. The word "probability" means how many times an experiment can be repeated with the same results. (For example, there is a fifty-fifty chance of a penny landing heads or tails in a coin toss.) In biological stereology, objects (i.e. particles) in a volume are measured on sections through that volume. In general, a section is defined as the intersection between two spaces. After a random section is cut using a microtome, profiles and objects appear as geometrical elements.

In stereology, what is the chance of hitting an object in a section? One basic principle of stereology is that the larger the number of particles in a volume, the larger the number of profiles on a section through that volume. In addition, the larger the particle, the greater is the chance of a section containing its profile. The number of profiles, therefore, depends on both the number of particles per unit volume and particle size. All "two-dimensional" histologic sections also have a thickness. To ensure that only one profile per particle is measured, each section cut should be spaced at an

appropriate distance. For example, the distance for megakaryocytes is >50 μm, since most megakaryocytes are less than 50 μm in diameter.

Random sectioning

One of the foremost principles in stereology is random sectioning. Random sectioning must occur for the section to be representative of the volume from which it was taken. Assuming that the objects to be measured, i.e., megakaryocytes in bone marrow, are also randomly oriented, random sectioning creates an unbiased representation of megakaryocytes in bone marrow. Because one section would not usually be enough to represent an object, many sections must be made. In order for random sectioning to occur in the bone marrow, a plane (knife from a microtome) is passed through the volume to produce random profiles of the cells. The section, by definition, must be random with respect to the bone, i.e., top, middle and bottom must have the same likelihood of being sectioned, and with respect to the megakaryocytes within the bone. Megakaryocytes are not found in a fixed position in the bone marrow. Instead, megakaryocytes are housed near the sinusoids throughout the bone marrow, which are randomly oriented. Additionally, megakaryocyte stem cells and possibly whole intact megakaryocytes migrate from the bone marrow to the circulatory system (Tavassoli et al., 1980); therefore, this movement causes random orientation.

Measurement of cell profile area

Delesse was a French geologist who first showed, in 1847, that the percent of an object in a volume (volume density) was equivalent to the percent of that object on a section through the volume (areal density) (Delesse, 1847). Planimetry in stereology is based on the principle of Delesse.

Areas of object profiles in section can be measured in one of three ways: planimetry, linear integration, or point counting. All three methods have been proven successful; however, the point counting method has been found to be the easiest to use. In addition, both planimetry and linear integration require complex instrumentation not required using point counting methods. Stereological principles can be applied using either simple or complex instruments. Point counting methods have been employed using nothing but a transparent grid or a microscope eyepiece with graticules. However, computer-assisted point counting and computer-assisted tracing devices greatly decrease the time of collecting and analyzing data.

Techniques of profile measurement also differ in precision (Hilliard and Cahn, 1961). Based on the coefficient of variation (CV) for repetitive trials of a given experiment, the method of planimetry by the cut-out-and-weigh technique (which traces the profiles on photographic paper, cuts them out, and weighs them) was shown to be the most precise method, with a CV for repeated trails of 2.64%.

However, with an average time of 461 seconds, it was also the most time consuming of all the methods used. Like the cut-out-and-weigh method, circle fitting and polar planimetry were also precise, producing coefficients of variation of 3.32% and 3.84% respectively. However, the average times were long: 148 seconds for circle fitting and 323 seconds for polar planimetry. Line integration had the worst CV and a reasonably average time. The best method, which had a reasonable CV (4.74%) and a short average time (73 seconds) was point counting using a systematic lattice. Thus, taking into account the CV and average measurement time, the point counting method was shown to be the method of choice.

Point counting volumetry

Definition of method. Delesse showed that the volume density (volume fraction) of elements in the rock equals the areal density (areal fraction) of elements in a section through the rock. Delesse sliced a rock into sections with a thickness of dy and then measured the profile areas of elements in the rock. He reasoned that the volume of elements in the rock (V_e) equaled the summed products of element area and section thickness ($\Sigma A_e dy$), and that the total volume of the rock (V_T) equaled the summed products of total section area

and section thickness ($\Sigma A_T dy$). Because section thickness was a constant, $A_A = V_V$ as shown in equation 1.

$$\Sigma A_e dy / \Sigma A_T dy = V_e / V_T \qquad (1)$$

The Delesse priciple can be used for biological sciences (Webiel, 1979) as well as geological sciences (Delesse,1847). Using the spleen as an example, the A_A of the splenic components in a section can be shown to equal the V_V using the Delesse principle. Also, the A_A can be shown to equal the point fraction (P_P), as determined by point counting volumetry. For this proof, the spleen was sliced into 3 µm thick sections. If a photomicrograph of the spleen section is cut into small squares of equal area, the number of squares containing a given element or object is proportional to the total profile area of that object in the spleen section. Similarly, instead of cutting the spleen section into squares, a point set can be used. A transparent grid of equidistant test points applied to a section shows that the number of points falling on profiles of an object divided by total points on the section (P_P) is equal to the object area fraction in that section (A_A). Since $P_P = A_A$ and $A_A = V_V$, $P_P = V_V$.

Counting grids and point density

Single square lattice test systems have been used commonly for point counting. Double square lattice test systems where two test systems of different point density appear on the same grid, are used to measure two objects of widely differing V_V simultaneously. For example, both mitochondria and lysosomes in electron micrographs can be measured using two different point densities on the same grid.

In general, the point density of a grid must be designed so that no more than one point falls on the same profile as in equation 2 (Weibel, 1979):

$$d^2 > a_m \qquad (2)$$

d^2= one square lattice unit

a_m= largest profile area measured on a section

The total number of points required to measure an object was determined by the level of statistical precision for that object in a pilot study (Weibel, 1979). A 5 or 10% standard error was usually used as a reliable estimate of the points needed. Equation 3 has been used to estimate the points (P_C) needed for an object's (a) statistical precision:

$$P_C = t_\partial^{\,2} / d^2 * 1 - V_{Va}/V_{Va} \qquad (3)$$

Equation 4 estimated the d^2 in equation 3:

$$d^2 = (t_\partial * 1 - V_{Va}/P_a)^2 \qquad (4)$$

The $t_\partial^{\,2}$ value was the 90 to 95 % probability of being within a confidence interval with an error of 5 to 10%. Most of the time ∂ = 0.05 and t= 1.96 or ∂ =0.01 and t =2.57. The unbiased volume density of the object was estimated using a square lattice grid.

Numerical density and cell diameter

Two stereological methods have been commonly used to determine numerical density (N_V) and mean cell diameter (D): the Abercrombie/Dehoff and Rhines method (ADH) and the Weibel and Gomez method (WG). Mayhew compared these methods, which produce similar estimates of the numbers of organelles per unit volume of cytoplasm, on particles of known size and number (Mayhew et al., 1972). The WG method has some advantages over the ADH method. Shape changes were allowed in the WG method, while the ADH method assumed a spherical shape which may have produced biased results. The ADH method, a planimetry method, was more time consuming compared to the WG method, a point counting method (Weibel, 1979)

Method of Abercrombie/ DeHoff and Rhines.

A planimetry method has been proposed by Dehoff and Rhines to calculate the diameter and number of discrete particles. In this method, the number of cell profiles, i.e., megakaryocytes, per unit of tissue section area (N_A) was found to be related to the number of cells per unit of tissue volume (N_V) (Weibel, 1979). The profile diameters of megakaryocytes varied in bone marrow when cut. Each sectional cut through the megakaryocytes produced profile diameters ranging from d_{max} to near d_{zero}. The d_{max} represents an equatorial section and near d_{zero} a tangential section through a megakaryocyte. Using a computer program, frequency distributions of megakaryocyte profile diameters can be generated. The N_V can then be calculated from the N_A and profile diameters. The diameter averaged over all directions was defined as the mean caliper diameter (D). The D can be calculated by equation 5, where d equals mean section profile diameter. Thus, from estimates of N_A and D, the number of spherical megakaryocytes per unit volume (N_V) can be calculated, as shown by equation 6 (DeHoff and Rhines, 1961):

$$D=4(d/\pi) \qquad (5)$$

$$N_V= N_A/D \qquad (6)$$

Megakaryocyte profile diameters can be estimated using perimeter equivalent or area equivalent diameters. Calculation of profile diameter from perimeter (P), planimeteric area (A), and from area derived from measurements of major (a) and minor (b) axes are shown in equations 7, 8 and 9 respectively:

$$d=P/\pi \qquad (7)$$

$$d=2\sqrt{A/\pi} \qquad (8)$$

$$d=\sqrt{ab} \qquad (9)$$

The method of DeHoff and Rhines has been shown to be reliable for the estimation of N_V and D only if some common errors were eliminated. Significant errors were found to be caused by thick sections, biased cell shapes, and optically lost caps. Both D and N_A may be overestimated in thick sections. Abercrombie modified the DeHoff and Rhines equation 6 by correcting for thick sections, as shown by equation 10 (Abercrombie, 1949):

$$N_V=N_A/(D+t) \qquad (10)$$

t=tissue thickness

Weibel offered a derivation of the Abercrombie formula, shown below, and in Figure A3 for a "super slice".

$$N_W = N_V * A * t \quad (11)$$

$$N_0 = N_V * A * 2(D/2) \quad (12)$$

$$N = N_W + N_0 = N_V * a(t+D) \quad (13)$$

N_W=profiles with center inside the section volume

N_0=profiles with center outside the section volume

N_V= number of spherical particles per unit volume

N_A=number of spherical particles per unit area

A= area of section

D=particles diameter

t=thickness

N=true number of particles in secton

Because of the projected images of particles in a super slice, the N_A may be overestimation. Two types of profiles are formed in a super slice, profiles with centers inside the section volume and profiles with centers outside the section volume. Profiles are not formed if the profile centers are above the section. For a true estimate of the numbers of profiles in a super slice, Weibel reasoned that N equals the N_W plus N_0. Solving for N_V, equation 10

was generated by dividing equation 13 by the sectional area. As sectional thickness approached zero, $N_V=N_A/(D+t)$ was equivalent to $N_V=N_A/D$. Only the profiles with their centers in 't' or 'r' were used in estimating N_A as shown in Figure A3.

In addition, a correction for section thickness in estimating megakaryocyte number from bone marrow histological slides was first calculated by Harker (Harker, 1968) in equation 14:

$$N=N^1/(D/t+1) \qquad (14)$$

N=true number of cells in the bone marrow slides

N^1= the number of cells counted on the section area

After dividing both sides of the Harker equation by section volume (AT), $N/(AT)=N_V$ and $N^1/A=N_A$. Thus, Harker's equation 14 reduced to Abercrombie's equation 10.

Another source of error was the lost profiles called "optically lost caps". These profiles were lost because of problems in identifying small profiles in sections. Even if these small profiles could be identified, usually the error was high. Optically lost caps caused the underestimation of N_V and the overestimation of D. Equation 5 only worked well if the particle population was normally distributed. Most biological events are normally distributed; but due to optically lost caps, the remaining particles showed a skewed

distribution. This skewing affected the lower third of the profile diameter distribution of particles (Weibel, 1979). Weibel introduced a way of approximating optically lost profiles as shown by Figure A4:

1. Smooth the distribution of the peak.
2. Find the highest point or mode, of the distribution.
3. Draw a straight line one-half the mode to the zero point.
4. Draw lines up the y-axes every 3 μm with a midpoint 1.5 μm until the lines intersects the other acute line.

Finally, cell shape may have induced significant errors (Weibel, 1979). In the past, megakaryocytes were modeled as spheres. However, megakaryocytes are found to be more elliptical than spherical in the bone marrow, thus introducing a bias when using a spherical cell shape. The D of spheres and ellipsoids of equivalent volumes were compared on a graph in which the ratio of D ellipsoid/D spheres increased from 0 (ellipsoid axial ratios of 1:1) to 1.6 (ellipsoid axial ratios of 5:1). Diameters of ellipsoids with an axial ratio of 5:1 modeled after a spherical shape may, therefore, have a 60% error. DeHoff and Rhines created a nomogram, from which the ratio, and thus the degree of error, may be directly determined (DeHoff and Rhines, 1961).

Method of Weibel and Gomez

An alternative method has been proposed by Weibel and Gomez to calculate both N_V and D, which eliminates the need for correction factors for lost profiles and section thichness. If N_A and V_V were known, then the N_V of particles could be calculated by equation 15.

$$N_V = K/B \sqrt{(N_A^3 / V_V)} \qquad (15)$$

K=particle size distribution coefficient

B=particle shape distribution coefficient

V_V=Volume fraction of the particles

N_V=Number per unit volume

The value of K ranged between 1.02 and 1.1 for coefficients of variation between 0.1 and 0.3. The value of B ranged between 1.382 for a sphere (axial ratio =1:1) to 2.8 for an ellipse (axial ratio = 5:1). When N_A and V_V were used in a ratio, most of the section thickness effect was compensated because both N_A and V_V were overestimated in thick sections. Conversely, no correction is required for optically lost profiles since both N_A and V_V were underestimated.

3. MATERIALS AND MATERIALS

Experimental animals

Nine female C_3H mice were used in this study. The mice were seven weeks old and weighed 12-13 grams. Three mice were randomly assigned to each of three treatment groups: hypoxia, rabbit antimouse platelet serum (RAMPS) and control. Stimulation and inhibition of megakaryocytopoiesis were provided by rabbit antimouse platelet serum (RAMPS) and isobaric hypoxia at 5% ambient oxygen, respectively. The mice were kept in cages or hypoxia chambers and fed Purina ratchow and water. Mice were fed and watered and cages and hypoxia chambers were cleaned and changed twice weekly. Mice were kept in the animal facility of the University of Tennessee Veterinary Hospital, which maintains a constant temperature and humidity.

Experimental treatments

Preparation of RAMPS

RAMPS was prepared by the method of McDonald (McDonald et al., 1973c). Briefly, a sample of 100 ml of blood was collected into 3.8% sodium citrate solution by bleeding about 200 male C_3H mice. Blood was centrifuged at 800X G for 5 minutes and then the platelet button was washed in 1% ammonium oxalate. Also, the resuspended platelet button was washed two times in saline. The resuspended platelets

were put in a 5% suspension of water, and frozen. When the antiplatelet sera was needed, 1 ml was thawed, and was injected subcutaneously at different sites in a rabbit, two times per week for three weeks. Blood was collected from the ear vein and then processed into serum by removing the red and white blood cells, platelets, salts and fibrinogen. About 400 male C_3H mice were bled to obtain 200 ml of defibrinated blood. Defibrinated red blood cell recepters bind to most antibodies, except the antibodies to mouse platelets. The antiserum was heated for 30 minutes at 56°C to remove complements and then cooled. Absorbing RBC with antisera was done two more times with the same methods as described (McDonald et al., 1973c). Mice were given a single intraperitoneal injection of 0.1 ml of absorbed RAMPS to study the effects of stimulation of megakaryocytopoiesis. Three days after RAMPS injection, mice were killed by cervical dislocation.

Preparation of hypoxia condition

Mice were exposed to hypoxia for 14 days to study the effects of inhibition of megakaryocytopoiesis. To produce an hypoxic environment, the mice were placed in cages covered with dimethyl-silicone rubber membranes (McDonald and Lange, 1967) (Lange et al., 1968) (Lange et al., 1966), which are differentially permeable to oxygen and carbon dioxide. A partial pressure difference across the silicone rubber membrane was created when the mice breathed the oxygen in the cage. This difference caused the oxygen content in the

cage to be lower than the oxygen content outside the cage. Also the partial pressure difference caused a tolerable increase in carbon dioxide levels in the cages. The oxygen levels in the cages were 5-5.5 %, after eight hours of equilibration. The average oxygen level throughout the experiment was 5.2% as measured by a Brecker oxygen analyzer.

Tissue collection and preparation

Blood, bones and spleen

Blood was collected from the retroorbital sinus of each mouse to measure hematocrits, total red and white cell counts, platelet counts, and WBC differential counts. These parameters were used to assess and confirm the effects of the treatments on hematopoiesis. Standard manual hematologic techniques were used to measure hematocrits and platelet counts. However, an automatic particle counter was used to measure red and white cell counts. WBC differential counts were made from blood smears stained with Wright-Giemsa using an "Autotech". Mice were then killed by cervical dislocation and the sternum, humerus, femur, tibia, and the spleen were removed. Bones were washed and then fixed in 10% buffered formalin, decalcified and embedded longitudinally in Sorvol methacryalate embedding medium. The spleens were fixed in 10% buffered formalin and cut in half. The two halves were embedded in the same block of Sorvol methacralate embedding

medium. Sections of the bones and the spleens, 3μm thick, were cut at intervals of 75 μm and then stained with hematoxylin and eosin or methylene blue.

Smears

Because of the shortage of female C_3H mice, six $B_6D_2F_1$ mice were used to compare the smear method with the section method for effectiveness and usefulness in the quantitation of megakaryocyte size. After cervical dislocation, both femurs were removed from each $B_6D_2F_1$ mouse. One femur was fixed in 10% buffered formalin, decalcified and embedded longitudinally in Sorvol methacralate embedding medium. Sections of the bones, 3 μm thick were cut at intervals of 75 μm and then stained with hematoxylin and eosin or methylene blue. In the other femur of the same $B_6D_2F_1$ mouse, the bone marrow was extracted by the blow out method. In this method, the end of the bone was cut off and a hypodermic syringe filled with air was used to blow out the marrow. The bone marrow was mixed with plasma expander (0.5 ml of saline and 2 drops of heparin), and dispersed into a single cell suspension. Then the cell suspension was centrifuged at low a speed (100 X G), the blood cell button was pipetted and smeared onto acid-cleaned slides and then stained with Wright-Giemsa.

Data collection and analysis

Estimation of P_P, N_A, N_V and D.

The N_A and d of megakaryocyte profiles in bone marrow and spleen were made at a magnification of 1800 X on a light microscope, which was interfaced to a Zeiss Videoplan Image Analysis System. Each of the megakaryocyte profiles within the 0.018 mm^2 sampling field was measured for area, major and minor axes, and perimeter. Megakaryocyte profiles were not measured if they touched the right and lower borders of the field. This effect is known as the "edge effect" because the counting of profiles touching all edges of the field would lead to an obvious overestimation of N_A (Gunderson, 1977). A transparent sheet with 36 points was overlaid on each field for point counting. Sample points were located where lines intersected. The P_P of megakaryocyte profiles was used to estimate A_A and hence, V_V.

From a pilot experiment, d estimates from planimetric area measurements of megakaryocytes were statistically better than the estimates from either axis estimates or perimeter estimates. Areas of megakaryocytes were therefore used to generate frequency distribution histograms from the megkaryocyte profile diameters using equation 6 in this study. Histogram class sizes were 3 μm wide, and the mid point of each class was defined as the value for that class. Histograms were then corrected for lost profiles using the method shown in figure 10. After correcting for lost profiles called "optically

lost caps" and section thickness, equations 5 and 10 were used to calculate megakaryocyte D and N_V for the ADH method 1.

For methods 2 and 3, the Weibel and Gomez methods, the N_V was calculated first using equation 15 and then D was calculated using equation 6 solved for D. In equation 15, the N_A and V_V were calculated using point counting as shown by equation 16.

$$N_A = N/(P_T * d^2) \qquad (16)$$

$V_V = V_a/V_c$ = Points over object/Total points

Also, the coefficents B and K were used in equation 15. In method 2, the WG spherical model (WGS), B = 1.382 and K = 1.06; whereas, in method 3 the WG elliptical model (WGE), B =1.43 and k = 1.06. Megakaryocyte diameters on the smear preparations were calculated from planmetric area according to equation 8.

Due to shrinkage in tissue processing, a correction factor of 5% was applied to D and N_V values, using a microcomputer. This correction factor was an estimate based on comparing red blood cell diameters in tissue sections with mouse whole blood.

Statistical analysis

The statistical analysis was performed at the University of Tennessee using a general linear model (GLM) contained in the Statistical Analysis System (SAS) software. The results of megakaryocyte quantification methods one (ADH), two (WGS), and three

(WGE) were analyzed by analysis of variance. The differences among the means for the three treatment groups, nine mice, the four bones and the spleen were partitioned with a set of orthogonal contrasts for all section and smear preparations. In all analysis, differences having less than a 5% statistical probability of being due to chance ($p < 0.05$) were reported as significant differences. Also, the component of variance was calculated. Based on the statistical results, the most precise method was found using the coefficient of variation (CV) and the root mean square error (RMSE). The lower the CV and the RMSE, the higher the statistical reliability. The results of the white blood cell counts were analyzed by multivariate analysis of variance.

(WGE) were analyzed by analysis of variance. The differences among the means for the three treatment groups, nine mice, the four bones and the spleen were partitioned with a set of orthogonal contrasts for all section and smear preparations. In all analysis, differences having less than a 5% statistical probability of being due to chance ($p < 0.05$) were reported as significant differences. Also, the component of variance was calculated. Based on the statistical results, the most precise method was found using the coefficient of variation (CV) and the root mean square error (RMSE). The lower the CV and the RMSE, the higher the statistical reliability. The results of the white blood cell counts were analyzed by multivariate analysis of variance.

4. RESULTS

Review of materials and methods

Three female C_3H mice were randomly assigned to each of three treatment groups: hypoxia for 14 days, RAMPS for 3 days, or control. Inhibition and stimulation of megakaryocytopoiesis were provided by isobaric hypoxia at 5% ambient oxygen and rabbit antimouse platelet serum (RAMPS), respectively. After treatment, mice were killed by cervical dislocation and the sternum, humerus, femur, tibia and the spleen were removed, fixed and stained for light microscopy. Blood was also analyzed to evaluate the effects of treatments on common hematologic parameters. Megakaryocytes were quantified using a Zeiss Videoplan Image Analysis System, and megakaryocyte diameter (Dia) and number (Num) were calculated using three sterelogic methods:

Method 1. Abercrombie/DeHoff and Rhines: ADHDia (1A) and ADHNum (1B); megakaryocytes were modeled as spheres.

Method 2. Weibel and Gomez spheres: WGSDia (2A) and WGSNum (2B); megakaryocytes were modeled as spheres.

Method 3. Weibel and Gomez ellipses: WGEDia (3A) and WGEENum (3B); megakaryocytes were modeled as ellipsoids.

Effects of treatments on the peripheral blood

Table B1 shows the effects of hypoxia and RAMPS on the peripheral blood parameters measured. Hypoxia caused a significant decrease in platelet counts and a significant increase in hematocrit, total RBC and WBC counts. RAMPS also caused a significant decrease in platelet counts, and no significant difference in hematocrit or RBC counts or WBC counts as compared to the control.

Using logarithmic transformation of the numbers of lymphocytes, neutrophils, monocytes, and eosinophils, no significant differences were shown between the experimental group or the interaction group. However, significant differences were shown in the numbers of lymphocytes, neutrophils, monocytes, and eosinophils, between the three treatment groups as shown by Table B1.

Based on the SNK test for variability, the logarithmic means of lymphocytes differed significantly for each treatment group. The number of neutrophils and monocytes differed significantly only between the hypoxia treatment and control groups. The variability in neutrophils and monocytes was not significantly different between the RAMPS and control treatment groups. No significant differences were found for eosinophils between the treatment groups.

Effects of treatments on megakaryocytopoiesis

In general, megakaryocytopoiesis was stimulated by RAMPS and inhibited by hypoxia. As shown in table B2, treating mice with RAMPS produced a significant increase in both megakaryocyte diameter and number per unit volume; whereas treating mice with hypoxia for 14 days produced a significant increase in megakaryocyte diameter and a significant decrease in number as compared to the control. Megakaryocytes were found in the bone marrow and in the spleen. The relative diameters of the megakaryocytes in the bone marrow were similar to the diameters in the spleen. The relative number of megakaryocytes in the spleen was significantly less than the number in the bone marrow.

Differences between methods

Calculations of megakaryocyte diameter and number per unit volume by the quantitative methods used in this study are shown in Table B2 and B3. Using the WGSDia (2A) method or the WGEDia (3A) method, the mean diameters of the megakaryocytes were consistently higher than the mean diameters using the ADHDia (1A) method. Also, the number of megakaryocytes was lower using methods WGSNum (2B) or WSENum (3B) than using the ADHNum (1B) method.

In general, the WG methods 2 and 3 produced similar results for both diameter and number. The WGSDia (2A) and WGSNum (2B)

values for diameter and number of megakaryocytes were not significantly different from the values for WGEDia (3A) and WGENum (3B) respectively. Both of the WG methods 2 and 3 have similar root mean square error (RMSE) and coeffient of variation (CV) values for diameter and number. All of the methods for measuring diameter--ADHDia (1A), WGSDia (2A) and WGEEDia (3A)--had similar RMSE and CV values. Similarly, all of the methods for measuring number--ADHNum (1B), WGSNum (2B), and WGENum (3B)--had similar RMSE and CV values as shown in Tables B4 and B5.

Smear verses section preparations

Under normal conditons in section preparations, megakaryocytes averaged 25 μm (0.08) (ADH); whereas, under normal conditions in smear preparations, megakaryocyte diameter averaged 40 μm (0.17) as shown in Table B6.

5. DISCUSSION

Significance of the study

Many investigators have studied altered megakaryocytopoiesis using megakaryocyte diameter and number as a measure of maturity and population, respectively (O'dell et al., 1970) (MacPherson, 1974) (Penington, 1979) (McDonald et al., 1978) (Enomoto et al., 1980) (McDonald et al., 1983). In general, the size and number of megakaryocytes reported by different investigators were similar, except for minor variation due to differences in quantitative method.

In previous experiments associated with altered megakaryocytopoiesis, some investigators have not made a statistically reliable estimate of the number of profiles in different hematopoietic organs, i.e., bone marrow and spleen. In addition, some investigators did not allow for optically lost profiles or the addition of projected images of the cell contained within the section volume in paraffin sections. It has been shown that due to tissue shrinkage a 15% error could occur. Therefore, a correction factor for shrinkage should be used in calculating megakaryocyte number and diameter. The use of plastic sections reduced the shrinkage to 5% (Cullen and McDonald, 1986). The use of plastic sections instead of paraffin sections may be useful in future studies on discrete particles like megakaryocytes. In this study, many of these errors were corrected by using the principles of stereology. Additionally, investigators can not directly compare results from one laboratory

to another, because of different counting procedures or not defining the area of field sampled. Some of the variables in quantitating megakaryocyte diameter and number could be reduced by using the principles of stereology as shown in this paper. Assays could be developed to quantitate conditions that mimic hypoxia. These hypoxic conditions included anemia, some types of cardiopulmonary insufficiency and altered oxygen tension in space travel and deep sea diving. These assays of megakaryocytes could lead to a better understanding of the hypoxia condition.

Thrombocytosis, thrombocytopenia, Hodgkin's disease, chronic myeloid leukemia, and lymphomas are all associated with an increase in TSF. A positive dose response of TSF has been shown for increasing diameter and number of megakaryocytes. Therefore, a new assay can be developed to quantitate the amount of TSF by calculating megakaryocyte diameter and number.

Effects of hypoxia on peripheral blood counts

Previous experiments have shown that hypoxia causes a significant increase in hematocrit values and RBC counts and a significant decrease in platelet counts (McDonald and Clift, 1976) (Langdon and McDonald, 1977) (Petursson and Chervenick, 1987). Several explanations for the decreased platelet counts have been proposed, including an increase in blood volume (Lail et al., 1970), splenomegaly (Penny et al., 1966) and stem cell competition (Birks et al., 1975) (McDonald and Clift, 1976) (Langdon and McDonald,

1977). An increase in blood volume (RBC and plasma) would dilute the platelets, thus causing the platelet count to be lower. An increase in blood volume was noticed, but it was not enough to reduce the platelet count significantly (McDonald and Clift, 1976) (Langdon and McDonald, 1977). A decrease in the platelet count was also noticed when hypoxia caused splenomegaly, suggesting that platelets were trapped in an enlarged spleen. When the spleen of a hypoxic mouse was removed, a greater reduction of platelet production was found than in splenctomized mice breathing ambient air. However, when one compared splenectomized-hypoxic mice to normal hypoxic mice, the reduction of platelets was not as great in splenectomized-hypoxic mice. Normal hypoxic mice had a higher platelet count. Thus, splenomegaly could not have accounted for all of the effect of hypoxia in the mice. It was estimated that a reduction in the platelet count due to splenomegaly and spleen trapping of platelets accounted for half the reduction of the platelet population (Langdon and McDonald, 1977).

The stem cell competition theory suggests that during the stimulation of erythropoiesis by hypoxia, fewer stem cells are available to form megakaryocytes (Birks et al., 1975) (McDonald and Clift, 1976) (Langdon and McDonald, 1977). This theory is based on several observations. The nature of the hypoxic condition was to lower the oxygen level to about 5% ambient air (McDonald and Clift, 1967) (Lange et al., 1966) (Lange et al., 1968). This triggered production of the hormone erythropoietin, which increased red blood

cell (RBC) production (McDonald et al., 1970a) (McDonald et al., 1970b). McDonald used the % S^{35} isotope to measure the platelet production during hypoxia (McDonald et al., 1970a) (McDonald et al., 1970b). Results showed that both platelet production and platelet counts were lower. Since the RBC production increased while platelet production decreased, strong evidence supported the stem cell competition theory (McDonald and Clift, 1976) (Langdon and McDonald, 1977). In another experiment, Balb/c mice were used, which had a defect in their erythropoietin production (McDonald and Clift, 1976). In a hypoxic condition, Balb/c mice had significantly higher platelet counts and only a slight increase in RBC counts compared to C_3H mice, which showed significant increases in RBC counts and thrombocytopenia. If hypoxia directly interrupted thrombopoiesis, then the Balb/c mice should have had a significantly reduced platelet count. The stem-cell theory was also tested in other experiments using RBC-transfused mice (McDonald et al., 1978). When RBC's were transfused into the mice, the level of erythropoietin was reduced. The same results were achieved using the transfused RBC mice as in the experiment using the Balb/c mice. In both experiments, lower levels of erythropoietin seem to be associated with normal platelet counts, thus supporting the stem-cell competition theory between megakaryocyte and erythrocyte cell lines. Balb/c mice produced little or no erythropoietin while their platelet production remained normal.

However, C_3H mice, with normal erythropoietin production in the hypoxia condition, showed increased erythropoietin levels and decreased platelet counts. Thus, an increase in erythropoietin levels suggested an increase in RBC production at the expense of platelet production.

In the author's experiment, both the hematocrit and RBC values after hypoxia were significantly higher, while the platelet counts were significantly lower as compared to the control values. These results confirmed the observations of previous investigators (Petursson et al., 1987) (McDonald and Clift, 1976) (Langdon and McDonald, 1977), and support the stem cell competition theory.

In a previous experiment, a five-fold increase in the WBC count was observed at the sixth day of hypoxia, followed by a thirteen-fold increase at the tenth day (Petursson et al., 1987). Similarly, a five-fold increase of WBC was found in the author's experiment after fourteen days of hypoxia. However, McDonald found a significant decrease in the WBC counts after one day hypoxia (McDonald, 1973c). Thus, this decrease and then an increase may suggest a biphasic mechanism in WBC production over a period of time in a hypoxic condition.

In addition to total WBC counts, a white cell differential count was performed to determine the effect of hypoxia on individual white blood cell types for each treatment group. Four types of white blood cells were indentified: lymphocytes, neutrophils, monocytes,

and eosinophils. Both the percentage and absolute number-per-volume of each cell type were considered. The data were converted into logarithms, because the WBC counts showed a skewed distribution. Because of greater statistical precision, a multivariate test was used to analze the data. The multivariate test checked for differences between experimental groups (EXP), treatment groups (TRT), and EXP*TRT (interaction). Under the hypoxic condition, the number of lymphocytes, neutrophils, and monocytes were significantly increased as compared to the control. Because of the small numbers of eosinophils counted, no significant increase was observed. It has been suggested that a multi-CSF could increase RBC colonies (Prystowsky et al., 1983) and myeloid colonies (Ihle et al., 1981) in vitro. EPO or a multi-CFS may be stimulating both the red and the white stem cell lines at the expense of the megakaryocyte cell line. In short-term hypoxia (one day), the cause of the low WBC count is not known; however, the leukocytosis of prolonged hypoxia may be due to stem cell competition (Petursson et al., 1987).

Effects of RAMPS on peripheral blood counts

Previous experiments have shown that an injection of RAMPS into mice causes a significant decrease in the platelet count (Jackson, 1973) (McDonald, 1978). It has been suggested that RAMPS affects platelets directly (McDonald, 1978) or indirectly (Stahl,et al., 1986) (Zucker-Franklin et al., 1986). Directly, RAMPS

binds to platelet surfaces, causing platelets to be aggregated, followed by release of ADP and MPS (mucopolysaccharides) from platelets in vivo (McDonald and Clift, 1976). Finally, labeled ^{35}S-MPS platelets are removed from the circulatory system, and found in the kidney (McDonald and Clift, 1976). Indirectly, RAMPS has been shown to bind to the surfaces of megakaryocytes 50% of the time in mice (Stahl et al., 1986). Because of incomplete antigenic cross-reactivitity with RAMPS, the other 50% of the megakaryocyte population does not bind. Human megakaryocytes from patients with idopathic thrombocytopenia purpura (ITP) showed 50% binding of RAMPS to cell surfaces. Reactive megakaryocytes with RAMPS showed an altered demarcation membrane system (DMS) near the peripheral edges. Whereas, nonreactive megakaryocytes showed no changes in the DMS near the peripheral edges. It has been suggested that only the more mature megakaryocytes were effected by RAMPS (Zucker-Franklin et al, 1986). Since the DMS is thought to be a storage pool for platelet membranes (Paulus et al., 1979), platelet membranes may be altered before platelet release. After release, platelets aggregated, released ADP and were finally removed from the circulatory system resulting in thrombocytopenia (McDonald and Clift, 1976). Thrombocytopenia caused an increase in TSF resulting in an increase in both megakaryocyte size and number (McDonald and Kalmaz, 1982).

Although causing severe thrombocytopenia; RAMPS has been shown to have no significant effect on hematocrits, and RBC and

WBC counts (McDonald, 1978). In the authors experiment, RAMPS caused a significant decrease in the platelet counts; whereas, no significant effect was found on the hematocrits, RBC and WBC counts.

Effects of treatments of megakaryocyte diameter and number

In mice, the spleen, as well as the bone marrow has been identified as an active site of hematopoiesis (Till and McCulloch, 1961). In the authors study, megakaryocytes were found in both locations, however, the number of megakaryocytes in the spleen was found to be a third of that found in the bone marrow of the control animals. This finding confirms previous observations (Cullen and McDonald, 1986). Due to the lower concentation of megakaryocytes, spleens may have to be sectioned 15 or 20 times for statistically reliable results in megakaryocyte quantitation; whereas the bone marrow may require only one or two sections for statistically reliable results. The RMSE and the CV determined the minimal number of profiles to be used. The lower the RMSE and CV, the more precise the statistical reliability. Four-hundred megakaryocyte profiles were used for statistically reliable results in the bone marrow. However, due to the low number of megakaryocyte profiles in the spleen and to time restraints, only 250 megakaryocyte profiles were used. This reduction in spleen megakaryocyte profiles caused only minimum error in the estimates of diameter and number per unit volume.

In a previous experiment, a two to three-fold increase in the number of megakaryocytes was observed in the spleen with a peak number found at the sixth day of hypoxia (Petursson and Chervenick, 1987). However, the number of megakaryocytes slowly decreased from the sixth day to the tenth day of hypoxia. Two mechanisms have been proposed to account for this increased number of megakaryocytes in the spleen at the sixth day of hypoxia.

1. The migration of the committed megakaryocyte stem cells and possibly whole megakaryocytes from the bone marrow to the spleen.
2. A better microenvironment in the spleen caused by changes in a regulatory hormone (a modified view of the stem cell competition theory).

It has been suggested that whole megakaryocytes migrate through bone marrow sinuses into the circulatory system (Tavassoli et al., 1980) and then migrate to the spleen (Petursson and Chervenick, 1987). Electron microscopic evidence showed megakaryocytes passing through a space 6 μm in diameter (Tavassoli et al., 1980). Most megakaryocytes are larger than 6 μm in diameter. However, migrating myeloblasts and erythrocytes conform to the size of an aperture of 3 μm (Lichtman et al., 1970) (Leblond et al., 1971). It has, therefore, been suggested that the cytoplasm of megakaryocytes can conform to these apertures in the parajunctional area of the sinusoidal endothelial cell (Tavassoli et

al., 1980). Additionally, it has been proposed that EPO may cause the displacement of adventitial reticular cells on the outside surface of the sinusoids (LeBlond et al., 1975). Thus, this displacement of adventitial reticular cells may enhance the migration of WBC and RBC (Aoki and Tavassoli, 1980) (LeBlond et al., 1975).

In the present experiment, a three-fold decrease was observed in the number of megakaryocytes in the spleen at 14 days of hypoxia using stereologic methods. This decrease may be explained by decreased migration of the committed megakaryocyte stem cells or by a modified stem cell competition theory. The stem cell migrational pool could have been depleted by the sixth day, causing a decrease in the number of megakaryocytes. Similarly, the stem cell competition theory may account for this decrease in the number of megakaryocytes in the spleen. Both the WBC and the RBC lines may be linked together, and when stimulated would lower the production of megakaryocytes. Human EPO or a multi-CFS (interleukin 3) may be stimulating both RBC and WBC production. In in vitro studies, a multi-CSF has been shown to promote RBC colonies (Prystowsky et al., 1983), as well as promote megakaryocyte and myeloid colonies (Ihle et al., 1981). Interleukin 3 (IL-3), a lymphokine, is made from activated T-helper lymphocytes (Weinstein et al., 1977) (Ihle et al., 1981). Additionally, IL-3 and Meg-CSF have similar molecular weights. Both Meg-CSF and IL-3 stimulated activities were inhibited by rabbit anti-Meg-CSF serum.

Thus, it has been suggested that IL-3 and Meg-CSF are the same molecules (Dukes et al., 1986). Similarly, EPO has been suggested to be a Meg-CSF (McLeod et al., 1976) (Williams et al., 1984). Conflicting evidence has been shown for human EPO stimulating megakaryocyte colonies. Previously, human EPO from urine stimulated megakaryocytes only 25% of the time in in vitro studies (Dukes et al., 1986). However, in the same study, in vitro experiments showed recombinant EPO (r-EPO) stimulating megakaryocyte colonies twice as much as human EPO from urine, as well as stimulating an increase in megakaryocyte precursor cells (Dukes et al., 1986). It has been suggested that human EPO may contain impurities (other plasma and urinary proteins) which may have inhibited the full effect of EPO to stimulate megakaryocytes in vitro and in vivo (Dukes et al., 1986). Since the level of Meg-CSF varied with different diseases, Hoffman has suggested that Meg-CSF may have a physiological role in vivo (Hoffman et al., 1981).

In RAMPS treated mice, both megakaryocyte number and size were increased in 14-day hypoxia, suggesting an increase in population growth (number) and maturation (size) respectively. Thus, an increase in growth of megakaryocytes suggested an increase in committed megakaryocyte stem cells (McDonald et al., 1982). Also in the authors experiment, RAMPS increased both the number and diameter of megakaryocytes following thrombocytopenia and thrombopoiesis.

Differences between quantitation methods

The WGS and WGE methods were devised and applied in theory and practice differently from the ADH method for calculating megakaryocyte diameter and number. In the WG methods, 2 and 3, point counting was used to estimate both N_A and V_V. The only difference between the WG methods was the shape constant, B. Method 2 assumed a spherical model; whereas method 3 allowed for an ellipoidal shape. Since megakaryocytes in this study were nearly spherical, methods 2 and 3 produced similar estimates for both megakaryocyte number and diameter.

In the WG methods the diameter values were consistently higher and the number values consistently lower than the ADH values for diameter and number. These results confirm those of an earlier study, where higher values for diameter and lower numbers seem to be associated with the WG methods (2A, 2B and 3A, 3B) when compared to the ADH method (1A and1B) (Cullen and McDonald, 1986). Lower number in the WGS and the WGE methods compared to the ADH method may be explained by the inability to identify small megakaryocyte profiles. The ADH method allowed for the approximation of optically lost profiles; whereas, the WGS and WGE methods does not allow for this correction. Higher diameter in the WGS and WGE methods compared to the ADH method may be explained by projected images of particles in a super slice, which overestimates the diameter.

Also, the diameter and number for the WGS method and the WGE method may be overestimated because of low numbers of fields counted. WG methods 2 and 3 have been shown in previous experiments to require counting more than 400 profiles for a higher statistical reliability (Cullen and McDonald, 1986). The author's present results confirmed this. Similar results of diameter and number of megakaryocytes were obtained by the author in additional experiments for both hypoxia and RAMPS treatments, with twice as many profiles counted. Predictably, the RMSE and the CV were lower in the author's additional experiment than the original experiment. Hence, the addition of profiles to the analysis produced results that were more statistically precise.

Comparing the precision of the methods

For all treatment groups pooled together, the most precise methods for mean diameter and for number were ADHDia (1A) and WGEENum (3B). Examination of each treatment group separately allowed the analysis of the efficiency of a method on a certain treatment group. In both the hypoxia and the RAMPS treatment , the most precise method for measuring diameter was method ADHDia (1A), while the most precise method for measuring number was WGSNum (2B). In the control, the most precise method for measuring diameter was WGEDia (3A) and for measuring number method WGENum (3B).

In the past, megakaryocytes have been modeled as spheres (Harker, 1968) (Jackson and Edwards, 1977) (Ebbe and Phalen, 1979) (McDonald et al., 1983); however, megakaryocytes have been shown to have a more elliposoidal shape than a spherical shape in the bone marrow. In the present study, control megakaryocytes were slightly more ellipsoidal than spherical; whereas, under the hypoxic and RAMPS treatments, megakaryocytes were nearly spherical in shape. Based on the RMSE and CV,in addition to the similarity of results obtained using the spherical and ellipsoidal methods, megakaryocytes can be modeled as spherical for all treatments (hypoxia, RAMPS and control) with minimum error.

Univariate analysis

The univariate analysis checked for differences in diameter and number of megakaryocytes between mouse-to-mouse, bone-to-bone, and for an interaction term (mouse *bone). There was no significant difference between mice in any of the three treatment groups except for method ADHDia (1A) which measured diameter in the hypoxic condition. Similarly, no interaction was observed in any of the treatment groups. However, bone-to-bone differences were observed for megakaryocyte numbers using a univariate analysis. For both the diameter and the number of megakaryocytes, bone to bone difference was analyzed using the Student-Newman-Keuls (SNK) test. The mean diameter of megakaryocytes for the four bones (sternum, femur, humerus, tibia)

and the spleen were not significantly different from one another using methods ADHDia (1A), WGSDia (2A), or WGEDia (3A). Except for the spleen, the relative numbers of megakaryocytes within the sternum, femur, humerus and the tibia were not significantly different using the three methods for number ADHNum (1B), WGSNum (2B) and WGENum (3B). The relative number of megakaryocytes in the spleen was significantly lower than in the bone marrow.

Smear verses section preparations

In some previous studies, megakaryocyte diameters have been measured on bone marrow smear preparations (Ebbe et al., 1981) (Shreiner et al., 1980). Due to the force of gravity and lack of connective tissue support, megakaryocytes are flattened and distorted when compared to those in section preparations.

In the authors study, under normal conditions, a 63% increase in the diameter of the megakaryocytes was found in smear preparations compared to the section preparations. If this increase in smear diameters is taken into account, megakaryocyte diameters in the smear and the section preparations may be interconverted.

Using the blow-out method for bone marrow extraction, the megakaryocyte numbers were greatly decreased when compared to the megakaryocyte numbers in the section preparations. It is possible that some of the megakaryocytes were destroyed, especially the larger megakaryocytes. Also, large and small megakaryocytes may not flatten out proportionately onto the slide.

Using stereologic methods, megakaryocyte numbers in smears can not be estimated, since by definition, stereology is a branch of mathematical methods based on geometric probability which estimates three-dimensional values of an entire structure from two-dimensional measurements on sections of the structure.

One of the major principles in stereology is that the sample must be representative of the object from which it was taken. In the authors experiment, smear preparations showed constant and reproducible results of the distribution curve for class sizes verses number of megakaryocytes. Thus, the blow-out method is representative of the megakaryocytes found in the smear preparations. However, because of the ease of quantifying smear preparations compared to section perparations, smear preparations may be the method of choice for quantifying megakaryocyte diameter.

REFERENCES

Abercrombie M, (1946) Estimation of nuclear population from microtome sections. Anat Rec 94: 239-247.

Abildgaard CF, Simone JV (1967) Thrombopoiesis. Seminars in Hematology 4: 424-431.

Aoki M, Tavassoli M (1980) Red cell egress from bone marrow in state of transfusion plethora. Exp Hematol 9: 231-239.

Becker RP, DeDruyn PH (1976) The transmural passage of blood cells into myeloid sinusoids and the entry of platelets into the sinusoidal circulation: A scanning electron microscope investigation. Am J Anat 145: 183-206.

Birks JW, Klassen LW, and Gurney CW (1975) Hypoxia-induced thrombocytopenia in mice. J Lab Clin Med 86: 230-238.

Bizzozero G, Sul Midollodelle Ossa (1869) Seconda communicazione preventia. Zentrabl Med Wissenchr 10: 149-150.

Burstein SA, Adamson JW, Harker LA (1980) Immunologic stimulation of early murine hematopoiesis and its abrogation by cyclosporin A. J Cell Physiol 103: 201-208.

Chat LX, Ebbe S, Baldin M (1965) Essai de l'active thrombopoietique du filtrat de plasma chez le rats recepteurs. Nouv Rev Franc Hemat 3: 405-411.

Cooney DP, Blatt WF, Louis-ferdinand R, Smith BA (1965) Studies on the thrombopoiesis II. Effect of a non-dialyzable spleen preparation on the thrombocyte level. Scan J Haematol 2: 195-207.

Cooper GW (1970) The regulation of thrombopoiesis. In: Goron, A.S. (ed.) : Regulation of Hematopoiesis Appleton-century Crofts, New York. pp. 1611-1629.

Cooper GW and Cooper B (1972) The effect of exposure to reduced barometric pressure upon platelet incorporation of ^{35}S-sulfate and platelet level in mice. Scientific Program, 15th Annual Meeting of American Society of Hematology. p. 99 (abstr).

Cruz-Orive LM (1978) Particle size-shape distributions: the general spheroid problem II. Stochastic model and practical guide. J Microsc 112: 153-167.

Cullen WC and McDonald TP (1986) A comparison of stereologic techniques for the quantification of megakaryocyte size and number. Exp Hematol 14: 782-788.

Dassin E, Bourebia J, Najean Y, and Rossert AM (1983) Act Haematol 69: pp. 249-253.

De Gabriele G, and Pennington DG (1967) Regulation of platelet production: "Thrombopoietin". Brit J Haematol 13: 210-223.

DeHoff RT, Rhines FN (1961) Determination of the number of particles per unit volume from measurements made on randon plane section : the general cylinder and the ellipsoid. Trans AIME 221: 975-984.

Delesse MA (1847) Procede mecanique pour determiner la composition des roches. C.R. Acad. Sci. (Paris) 25: 544-550.

Donne A (1842) Compt rend Acad Sci 14: 366-368.

Dukes PP, Egrie JC, Strickland (1986) Meagakaryocyte colony stimulating activity of recombinant human and monkey erythropoietin. In: Levine R, William N, Levin J, Evatt B (eds.) Megakaryocyte Development and Function.105-109.

Ebbe S, Phalen E (1979) Does autoregulation of megakaryocytopoiesis occur? Blood Cells 5: 123-138.

Ebbe S, Phalen E, Stohlman F (1973) Abnormalities of megakaryocytes in W/W/mice. Blood 42: 857-864.

Ebbe S, Phalen E, Threatte G and Adrado C (1981) Megakaryocytopoiesis in irradiated splenectomized mice. Exp Hematol 9: 1020-1027.

Enomoto K, Kawakita M, Kishimoto S, Katayama N, and Miyake T (1980) Thrombopoiesis and megakaryocyte colony stimulating factor in urine of patients with aplastic anaema. Brit J Haematol 45: 551-556.

Erslev AJ, Gabuzda TG, (1979) In: Pathophysiology of Blood. Second ed. W.B. Saunders Company, Philadelphia.

Evatt BL, Levin J (1969) Measurement of thrombopoiesis in rabbits using ^{75}Selenomethionine. J Clin Invest 48: 1615-1626.

Evatt B, Levine J, and Alazy KM (1979) Partial purification of thrombopoietin from the plasma of thrombocytopenia rabbits. Blood 54: 377-388.

Freedman MH, McDonald TP, Saunder EF, (1981) Differentiation of murine marrow megakaryocyte pregenitors (CFU-M): Humoral control in vitro. Cell and Tissue Kinet 14: 53-58.

Gunderson HJG (1977) Notes on the estimation of the numerical density of arbitrary profiles: the edge effect. J Microsc 111: 219-223.

Harker LA (1968) Megakaryocyte quantitation. J Clin Invest 47: 452-457.

Harker LA (1974) Control of platelet production. Ann Rev Med 25: 383-400.

Harris PF, Harris RS, Kugler JH, (1966) Studies of the leukocyte compartment in guinea-pig bone marrow after acute haemorrhage and severe hypoxia: Evidence for a common stem-cell. Br J Haematol 12: 419-432.

Hill R, and Levin J (1986) Partial purification of thrombopoietin using lectin chromatography. Exp Hematol 14: 752-759.

Hilliard JE and Cahn JW (1961) An evalvation of procedure in quantitive metalography for volume fraction analysis. Trans Inst Min Engns 221: 344-353.

Hoffman R, Mazur E, Bruno E and Floyd V (1981) Assay of an activity in the serum of patients with disordors of thrombopoiesis that stimulates formation of megakaryocyte colonies. N Engl J Med 305

Hoffman R, Straneva J, Yang H, Bruno J, Brandt L, Geissler LU (1986) Exp Hematol 14: 417 (Abstr).

Hoffman R, Yang HH, Bruno E, Straneua JE (1985) Colony-stimuating factor from human plasma. J Clin Invest 75: 1174-1182.

Howell WH (1890) Observations upon the occurrence,structure, and function of the giant cells of the marrow. J Morphol 4: 117-130.

Howell WH, Donohue DD (1939) The production of blood platelets in the lungs. J Exp Med 65:171-203.

Ihle JN, Pepersack L, Rebar L: regulation T cell differentiation: (1981) In vitro induction of 20-hydroxysteroid dehydrogenase in splenic lymphocytes from athymic mice by a unique lymphokine. J Immunol 126: 2184.-2189.

Jackson CW (1973) Nature of platelet-agglutinating factor in serum of platient with idopathic thrombocytopenic purpura. Blood 42: 413-421.

Jackson CW, Edwards CC (1977) Biphasic thrombopoirtic responce to severe hypobaric hypoxia. Br J Haematol 35: 233-244.

Junqueira LC, and Carneriro J (1983) Basic Histology Fourth ed, Lange Medical Publications pp 3, 259-260,288-289.

Kalmaz GD, and McDonald TP (1981) In vitro cloning and cellular propereties. In: Evatt B, Jevine R, Williams N, Eds. : Megakaryocyte Biology and Precursors: Elsevier North Holland New York, NY. pp.77-86

Kalmaz GD, and McDonald TP (1982) Assay for thrombopoietin : A new, more sensitive method based on measurement of the small acetylcholinesterase-positive cell. Proc Soc Exp Biol Med 170: 213-219.

Kalmaz GD, and McDonald TP (1985) Effect of thrombopoietin on in vitro production of megakaryocytes from fetal mouse liver cells. Proc Soc Exp Biol Med 180: 50-56.

Keleman E (1979) Small megakaryocytes in human embryonic liver. Blood Cell 5: 101-102

Kimura H, Burstein SA, Thorniny D, Powell L, Harker LA, Fialkow PJ, Adamson JW (1984) Human megakaryocytic progenitors (CFU-M) assaying in methylcellulose: physical characteristics and requirements for growth. J Cell Physiol 188: 87-96.

Kinosita R, Ohno S (1961) Biodynamics of thrombopoiesis. In: Johnson SA,Monto RW, Rebuck JW, Horn RC, Eds. Blood Platelets Little Brown, Boston: 611-616.

Krizsa F., Cserhati, I., Halasa A., and Joo F., (1977) Incorporation of H^3-leucine in the mouse kidney in thrombocytopenia. Attempt to demonstrate thrombopoietin production. Acta Haematologica 58: 134-137.

Langdon JR, McDonald TP (1977) Effect of chronic hypoxia on platelet production in mice. Exp Hematol 5: 191-198.

Lange RD, Simmins ML, and Dibelius NR (1966) Polycythemic mice produced by hypoxia in silicone rubber membrane enclosures: a new technique. Proc Soc Exp Biol Med 122: 761-764.

Lange RD, Simmons ML, and McDonald TP (1968) Use of silicone rubber membrane enclosures for preparation of erythropoietin assay mice. Ann N.Y. Acad Sci 149: 34-39.

Lail S, McDonald TP, Lange RD (1970) Effect of hypoxia on body weight, body water, and hematological values of mice. Lab Animal Care 20: 483-488.

Layendecker SJ and McDonald TP (1982) The relative roles of the spleen and bone marrow in platelet production in mice. Exp Hematol 10: 332-342.

LeBlond PF, Celle LA, and Weed PL (1971) Cellular deformability: a possible determinant of the normal release of maturing erythrocytes from the bone marrow. Blood 37: 40-47.

LeBlond PF, Chamberlain JK, and Weed RI (1975) Scanning electron microscopy of erythropoietin-stimulated bone marrow. Blood Cells 1: 639-641.

Levin J, Levin FC, Hull DF III, and Penington DG (1982a) The effect of thrombopoietin on megakaryocyte-CFC, megakaryocyte and thrombopoiesis : with studies of ploidy and platelet size. Blood 60: 989-998.

Levin J, Levin FC, and Metcalf D (1982b) The effect of acute thrombocytopenia on megakaryocyte-CFC and granulocyte-macrophage-CFC in mice: Studies of bone marrow and spleen. Blood 56: 274-283.

Levine RF (1981) Criteria for the identification of megakaryocytes. In: Megakaryocyte biology and precursors. (Evatt B, Levin R, William R eds) Elsevier : North Holland, New York, p 203-207.

Levine RF, Hazzard KC, and Lamberg JD (1982) The significance of megakaryocyte size. Blood 60: 1122-1131.

Lichtman MA (1970) Cellular deformability during maturation of myeloblasts: a possible role in marrow egress. New Engl J Med 283: 942-948.

Linman JW, and Pierre RV (1964) Studies on the thrombocytosis-promoting effect of plasma and urine. An Int Med 60: 326-335.

Long MW, Williams, N (1981) Immature megakaryocytes in the mouse: morphology and quantitation by acetylcholinesterase staining. Blood 58: 1032-1039.

MacPherson GG (1974) Changes in megakaryocyte development following thrombocytopenia. Brit J Haematol 26:105-115.

Martin JF, Trowbridge EA, Salmon G, and Plumb J (1983) The biological significance of platelet volume: its relationship to bleeding time, platelet thromboxane B_2 production and megakaryocyte nuclear DNA concentration. Thromb Res 32: 443-460.

Mazur EM (1986) Megakaryocytopoiesis and platelet production: a Review. Exp Hematol 15: 340-350.

Mayhew TW (1972) A comparison of several methods for stereological determination of organelles per unit volume of cytoplasm. J Microscopy 46:37-44.

McDonald TP (1973a) Regulation of thrombopoiesis. Medicina 33: 459-466.

McDonald TP (1973b) The Hemagglutination-inhibition assay for thrombopoietin. Blood 41: 219-233.

McDonald T P (1973c) Bioassay for thrombopoietin utilizing mice in rebound-thrombocytosis. Proc Soc Exp Bio Med 144: 1006-1012.

McDonald TP (1973d) Bioassay for thrombopoietin utilizing mice in rebound-thromnocytosis. Proc Soc Exp Bio Med 144: 1006-1012.

McDonald TP (1976) A comparison of platelet size, platelet count, and platelet ^{35}S incorporation as assays for thrombopoietin. Br J Haematol 34: 257-267.

McDonald TP (1978) Platelet production in hypoxia and RBC-transfused mice. Scand J Haematol 20: 213-220.

McDonald TP (1980a) Effect of thrombopoietin on platelet size of mice. Exp Hematol 8: 527-532.

McDonald TP. (1980b) Annotation: Assay and site of production of TSF. Br J Haematol 49:493-499.

McDonald T P (1981) In Vitro Cloning and Cellular Properties, B Evatt, R Levine, and N Williams Eds., In : Megakaryocyte Biology and Precursors: Elsevier North Holland. New York, NY. pp. 39-57

McDonald TP, Clift R (1976) Mechanism of thrombocytopenia induced in mice by antiplatelet serum. Haemostasis 5: 38-50.

McDonald TP, Clift R, Cottrell M, and Long MN (1986) Further studies on the purification and assay of thrombopoietin In:RF Levine, N Williams, J Levin and BL Evatt Eds., Megakaryocyte Development and Function Alan R Liss, Inc., New York, NY. pp. 215-219.

McDonald TP, Clift R, Cottell M and Long MN (1987) Recovery of thrombopoietin during purification. Biochem Med and Metabol Biol. 37: 335-343.

McDonald T P , Cottrell M, and Clift R (1977) Hematologic changes and thrombopoietin production in mice after X-irradiation and platelet-specific antisera. Exp Hematol 5: 291-298.

McDonald TP, Cottrell M, Clift R, Khouri JA and Long MD (1985) Studies on the purification of thrombopoietin from kidney cell culture medium. J Lab Clin Med 106: 162-174.

McDonald TP and Kalmaz GD (1983) Effect of thrombopoietin on the number and diameter of marrow megakaryocytes in mice. Exp Hematol 11: 91-97.

McDonald TP, Lange RD (1967) Erythropoietin bioassay utilizing silicone rubber membrane enclosures. J Lab Clin Med 70: 48-56.

McDonald TP, Lange RD, and Kretchmar AL (1970a) Mode of action of erythropoietin in polycythemic mice. II. Response in mice with polycythemia induced by hypoxia or transfusion. J Lab Clin Med 77: 124-134.

McDonald TP, Lange RD, and Kretchmar AL (1970b) Mode of action of erythropoietin in polycythemic mice. II. Response in mice with polycythemia induced by hypoxia or transfusion. J Lab Clin Med 77: 134-143.

McLeod DL, Shreeve MM, Axelrad AA (1976) Induction of megakaryocyte colonies with platelet formation in vitro. Nature 261:492-494.

Minot GR (1922) Megakaryocytes in the peripheral circulation. J Exp Med 36: 1-7.

Mizoguchi H, Fujiwara Y, Sasaki R, Chibia H (1986) The effect of interleukin 3 and erthropoietin on murine megakaryocyte colony formation.In: RF Levine, N Williams, J Levin and BL Evatt Eds., Megakaryocyte Development and Function. Alan R Liss, Inc., New York, NY. pp. 111-115.

O'dell TT (1974) Platelets: Production, function, transfusion and storage. Baldin G, Ebbe S, Eds.: 11-20 Grune and Srattin, New York, NY. pp. 11-20

O'dell TT, Jackson CW, Friday TJ (1970) Megakaryocytopoiesis in rats with special reference to polyploidy. Blood 35:775-782.

O'dell TT, Jackson CW, Friday TJ, and Charsha DE (1969) Effects of thrombocytopenia in megakaryocytopoiesis. Brit J Haematol 17:91-101.

O'dell TT, McDonald TP, and Howsden FL (1964) Native and foreign stimulation of platelet production. J Lab Clin Med 64: 418-24.

O'dell TT, McDonald TP, Shelton C, and Clift R (1979) Stimulation of mouse megakaryocyte endomitosis by plasma from thrombocyopenic rats. Proc Soc Exp Bio Med 160: 263-265.

Ogle JW, Dunn CDR, McDonald TP and Lange RD (1978) The in vitro production of erythropoietin and thrombopoietin. Scan J Haematol 21: 188-196.

Paulus JM, Maigne J, and Keyhaani (1981) Mouse megakaryocytes secrete acetylcholinesterase. Blood 58:1100-1106.

Penington DG (1979) The cellular biology of megakaryocytes. Blood Cells 5: 5-10.

Penington DG, Olson TE (1970) Megakaryocytes in states of altered platelet production: cell number, size and DNA content. Brit J Haematol 18: 447-465.

Penny R, Rozenberg MC, and Firkin BG (1966) The splenic platelet pool. Blood 27: 1-16.

Petursson SR, Chervenick PA (1987) Effects of hypoxia on megakaryopoiesis and granulopoiesis. Eur J Haematol: 39: 267-273.

Prystowsky MB, Ihle JN, Rich I, Keller J, Otten G, Navjokos M, Loken M, Goldwasser E, Fitch FW (1983). Two biologically distinct colony stimulating factors are secreted by a T lymphocyte clone. J Cell Biochem 6: 37-44.

Queissner U., Queissner W., Spiertz B., (1971) Polyploidization of megakaryocytes in normal human with ITP and pernicious anaemia. Br. J. Haematol. 20: 489-501.

Radley JM, Scurfield G (1980) The mechanism of platelet release. BLood 56: 996-999.

Raha S, Wessmann W, and McDonald TP (1985) Isolation of mouse megakaryocyes: II. functional and metabolic aspects of two different maturational stages. Europ J Cell Biol 37: 111-116.

Rak K (1963) Regulation of platelet production Acta Med Acad Sci Hung 19: 67-81.

Robin C (1849) Sur l' existence de deux especes nouvelles d'elements anatomiques qui se trouvent dans le canal medullaire des os. Gaz Med de Paris 20: 992-993.

Schulman I, Abildgaad CF, Cornet JA, Simone JV, and Currimbhoy Z (1965) Studies of Thrombopoiesis II. Assay of human plasma thrombopoietin activity. J Pediatrics 66: 604-611.

Scurfield G, Radley JM (1981) Aspects of platelet formation and release. Am J Hematol 10: 285-296.

Shreiner DP, Weinberg J and Enoch D (1980) Plasma thrombopoietic activity in humans with normal and abnormal platelet counts. Blood 56: 183-188.

Siemensma NP, Bathal PS and Penington DG (1975) The effect of massive liver resection on platelet kinetics in the rat. J Lab Clin Med 86: 817-833.

Slater DN, Trowbridge EA, Martin JF (1983) The megakaryocyte in thrombocytopenia : A light and electron-microscope study supporting the theory that all platelets are produced in the lung. Thromb Res 31: 163-176.

Stahl CP, Zucker-Frankin D, and McDonald TP (1986) Incomplete antigenic cross reactivity between platelets and megakaryocytes: relevance to ITP. Blood 67: 421-428.

Steinberg B, Dietz AA, Atamer MA (1959) Mechanism of hematopoiesis-hematopoietic regulators in serum albumin. Ach Pathol 67: 496-504.

Tavassoli M and Aoki M (1980) Migration of entire megakaryocytes through the marrow blood barrier. Br J Haematol 48: 25-29.

Till JE and McCulloch, EA (1961) A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. Radiat Res 14, 213-224.

Trowbridge EA, Martin JF, Slater DN (1982) Evidence for a theory of physical fragmentation of megakaryocytes, implying that all platelets are produced in the pulmonary circulation. Thromb Res 28: 461-475.

Weibel ER (1979) Stereological Methods Vol 1. Academic Press. London.

Weinstein Y, Lindes HR, Eckstein B (1977). Thymus metabolizes progesterone, a possible enzymatic marker for T lymphocytes. Nature 266: 632-633.

Weintraub AH, Karpatin S (1974) Heterogeneity of rabbit platelets. II Use of the megakaryocytes to demonstrate a thromopoietic stimulus. J Lab Clin Med 83 : 896-901.

Williams N, Jackson H, Iscove N, and Dukes PP (1984) The role of erythropoitein ,TSF, and myeloid colony stimulating factors on murine megakaryocyte colony formation. Exp Hematol 12: 734-740.

Williams N, McDonald TP and Rabellino EM (1979) Maturation and regulation of megakaryocytopoiesis. Blood Cells 5 : 43-55.

Wright JH (1906) The origin and nature of the blood platelet. New Engl J Med 154: 643-645.

Zucali JR., McDonald TP., Gruber DF., and Mirand EA., (1977) Erythropoietin, thrombopoietin, and colony stimulating factor in fetal mouse liver culture media. Exp Hematol 5: 385-391.

Zucker-Frankin D, Stahl C, and Hyde P (1986) Antigenic dissimilarity between platelet and megakaryocyte surface membrane. In Megakaryocyte Development and Function pp. 259-264.

APPENDIXES

APPENDIX A

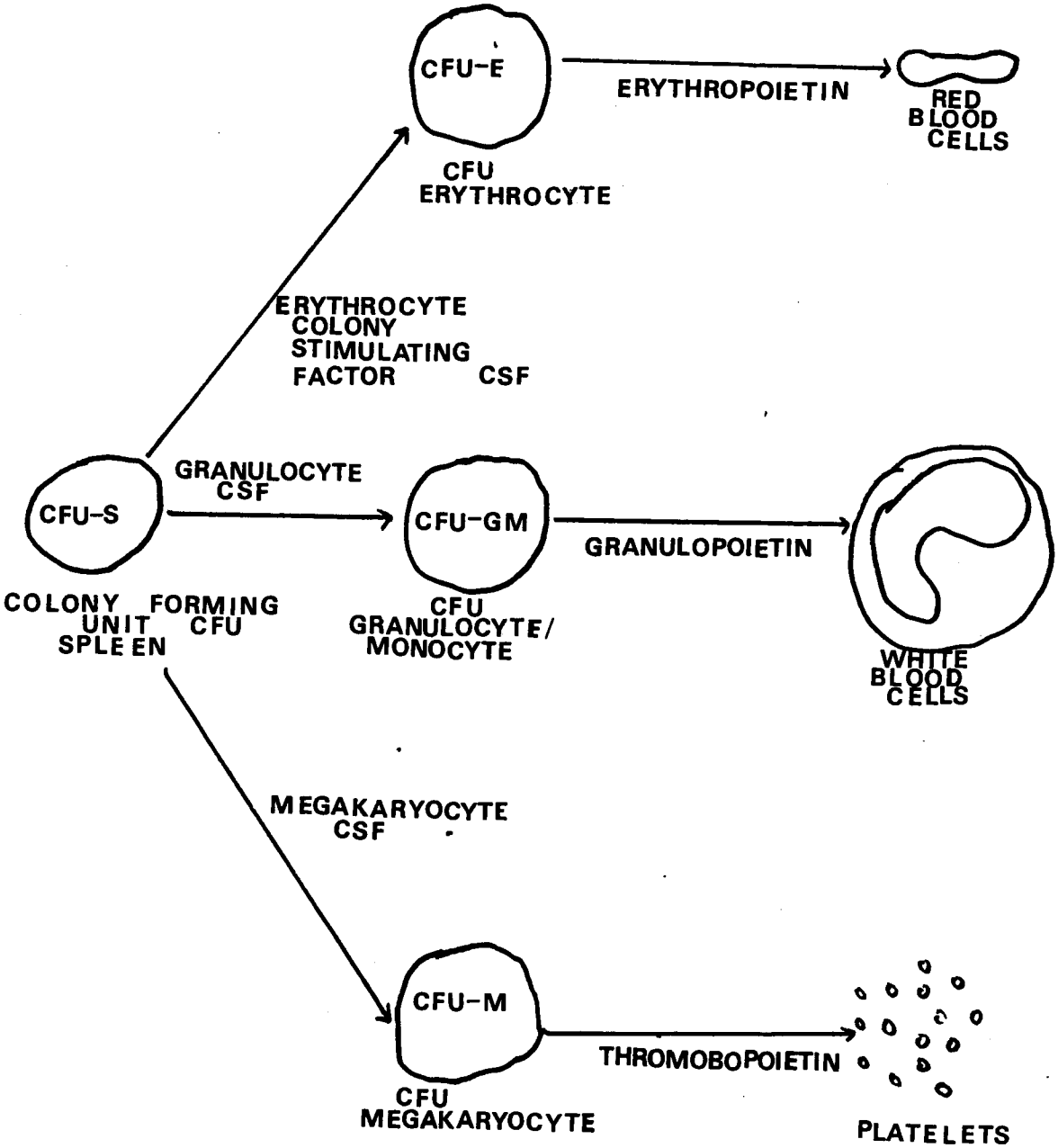

Figure A1. Model of hematopoiesis illustrating stem cell development.

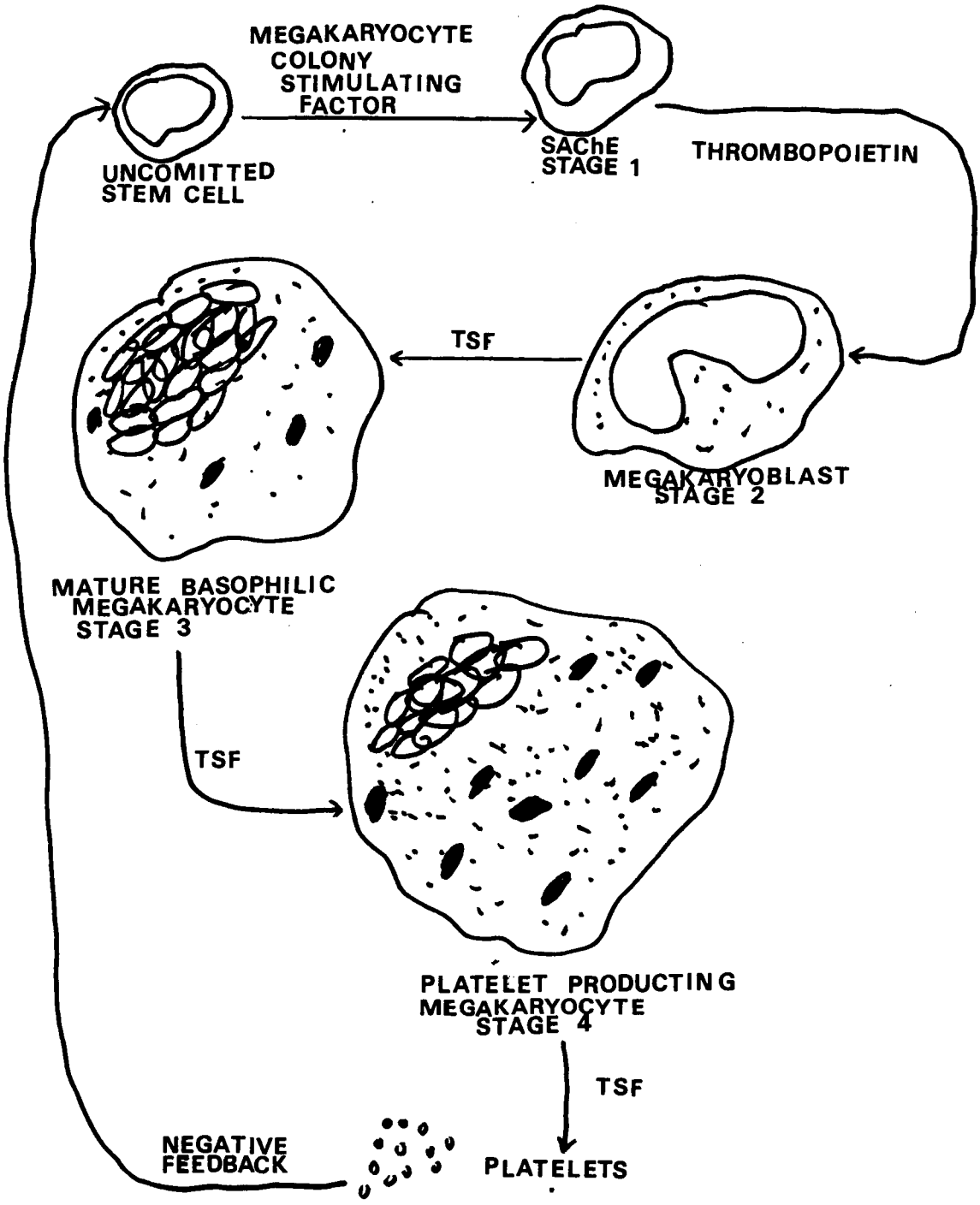

Figure A2. Model of megakaryocytopoiesis illustrating megakaryocyte development.

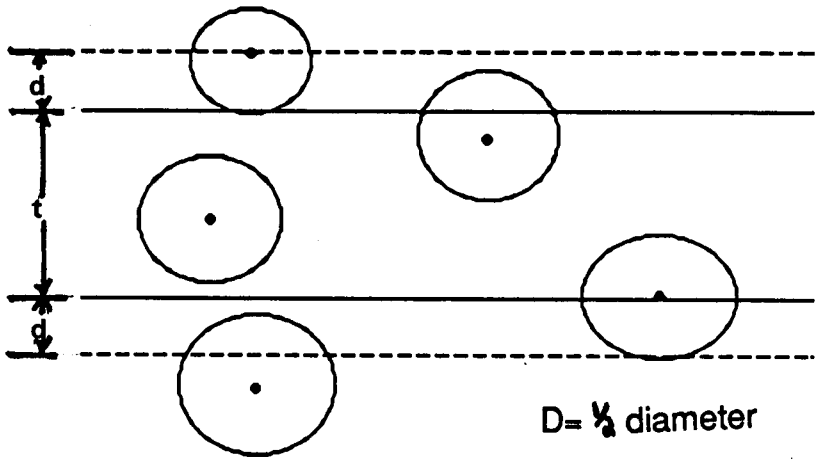

Figure A3. A histological section illustrating the correction for section thickness in the estimation of $N_{A.}$

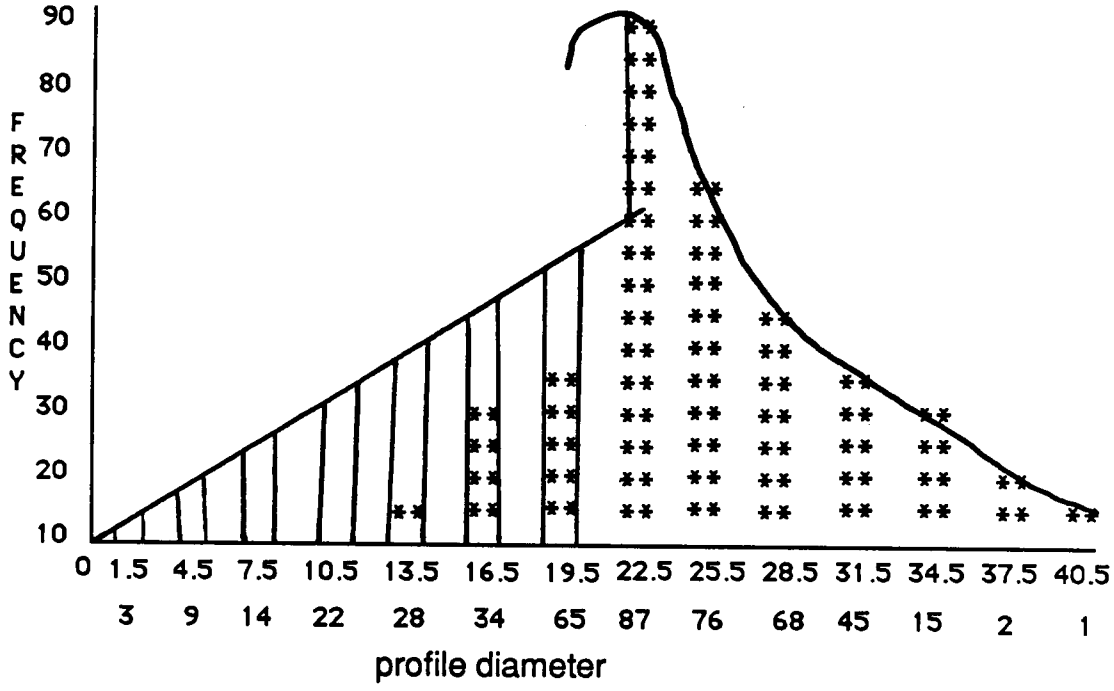

Figure A4. A megakaryocyte section profile diameter distribution illustrating the Weibel method for approximating optically lost profiles.

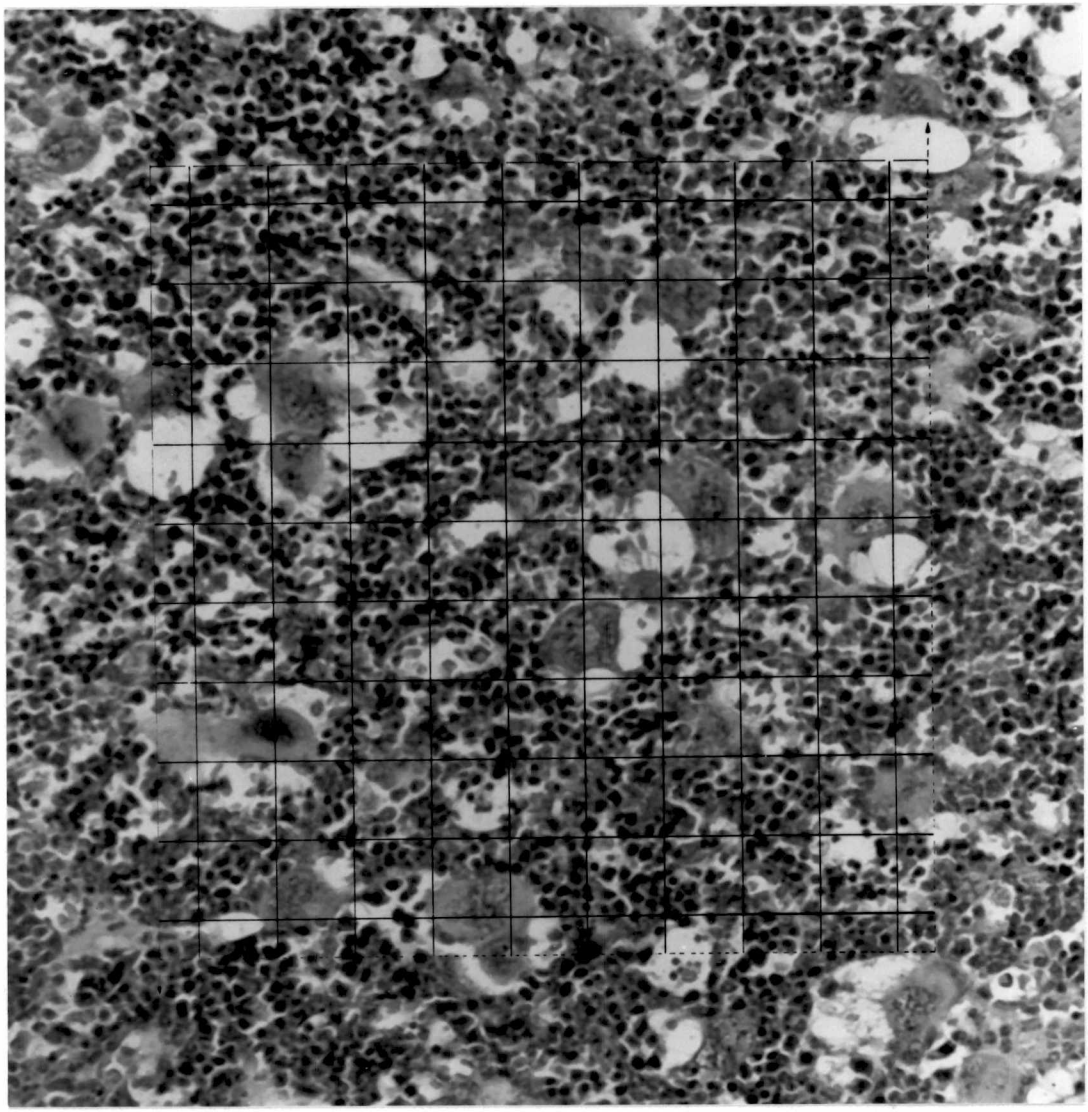

Figure A5. Sample field similar to that used in data collection and illustrating the Gunderson edge effect.

APPENDIX B

Table B1. The effects of rabbit antimouse platelet serum (RAMPS) and prolonged hypoxia on platelet, red and white cell counts, hematocrit and white blood cell differential counts in mice.

Parameter	Control		Hypoxia		RAMPS	
Hematocrit[a]	44.50	(0.28)	*79.00	(1.24)	44.30	(0.72)
Platlets[b]	11.03	(0.04)	*7.00	(1.04)	*5.62	(0.39)
Total RBC[c]	8.00	(0.17)	*11.50	(0.31)	7.80	(0.35)
Total WBC[d]	4.63	(0.29)	*28.63	(0.27)	6.09	(0.13)
WBC Differential[e]						
Lymphocyte	7.90	(0.04)	*9.91	(0.05)	8.38	(0.05)
Neutrophil	7.04	(0.04)	*9.91	(0.05)	7.41	(0.05)
Monocyte	3.88	(0.49)	* 5.68	(0.16)	3.89	(0.04)
Eosinophil	3.12	(0.41)	2.43	(0.96)	3.57	(0.49)

[a]expressed as % (SE)
[b]expressed as number/ $mm^3/10^5$ (SE)
[c]expressed as number/ $mm^3/10^6$ (SE)
[d]expressed as number/$mm^3/10^3$ (SE)
[e]expressed as Logarithms (SE) of % of each WBC type
* significantly different from the control value ($P < 0.05$)

TABLE B2. Mean megakaryocyte diameter and number per unit volume for all quantitative methods by treatment.

Methods		Dia		N_V	
		Hypoxia			
Abercrombie/ DeHoff and Rihines (ADH) spheres	Sternum	30.23	(0.20)	1225	(15.57)
	Femur	30.70	(0.30)	1308	(134.80)
	Humerus	31.27	(0.90)	1171	(83.75)
	Tibia	31.53	(0.60)	1066	(4.05)
	Spleen	30.07	(0.90)	101	(7.21)
Weibel and Gomez spheres (WGS)	Sternum	36.77	(0.20)	910	(59.50)
	Femur	34.70	(0.80)	862	(78.80)
	Humerus	35.83	(1.20)	946	(12.91)
	Tibia	35.17	(0.60)	838	(37.87)
	Spleen	34.43	(0.50)	84	(4.70)
Weibel and Gomez ellipses (WGE)	Sternum	35.35	(0.70)	956	(82.70)
	Femur	32.77	(1.10)	909	(69.48)
	Humerus	34.63	(1.20)	897	(15.82)
	Tibia	33.73	(0.50)	875	(33.82)
	Spleen	34.98	(0.50)	79	(4.58)
		RAMPS			
ADH	Sternum	30.80	(0.10)	4008	(255.00)
	Femur	30.00	(0.50)	5037	(148.54)
	Humerus	31.17	(0.40)	4377	(274.11)
	Tibia	30.70	(0.10)	3493	(465.00)
	Spleen	31.57	(0.50)	1022	(110.63)
WGS	Sternum	33.10	(0.52)	3142	(187.59)
	Femur	32.60	(1.15)	3411	(136.51)
	Humerus	33.77	(0.10)	3222	(230.07)
	Tibia	33.83	(0.30)	2661	(181.40)
	Spleen	31.67	(0.30)	863	(74.00)

Table B2 (Continued)

Methods		Dia RAMPS		N_V	
	Sternum	33.07	(0.57)	3247	(196.96)
WGE	Femur	32.07	(0.91)	3546	(160.55)
	Humerus	33.27	(0.15)	3275	(246.81)
	Tibia	32.73	(0.56)	2939	(162.92)
	Spleen	32.60	(0.68)	814	(36.69)
		Control			
	Sternum	25.83	(0.20)	3203	(210.77)
ADH	Femur	25.93	(0.15)	3167	(141.79)
	Humerus	25.98	(0.06)	3526	(556.13)
	Tibia	25.33	(0.38)	3630	(348.17)
	Spleen	26.97	(0.50)	1144	(24.66)
	Sternum	30.70	(0.06)	2455	(487.03)
WGS	Femur	29.83	(0.27)	2358	(170.84)
	Humerus	29.93	(0.32)	2567	(316.78)
	Tibia	30.30	(0.12)	2415	(124.93)
	Spleen	32.83	(0.64)	922	(14.62)
	Sternum	31.10	(0.06)	2204	(43.75)
WGE	Femur	30.23	(0.27)	2322	(97.54)
	Humerus	30.33	(0.32)	2537	(311.13)
	Tibia	30.60	(0.21)	2392	(127.64)
	Spleen	32.33	(0.47)	912	(13.89)

TABLE B3. RMSE and CV of megakaryocye quantitative methods for all treatments pooled together.

	RMSE	CV
DHDia (1A)	0.06700012	2.22852
WGSDia (2A)	0.09728391	2.94860
WGEEDia(3A)	0.09423532	2.81230
DHNum (1B)	443.93806609	17.7348
WGSNum (2B)	313.24313249	16.7911
WGEENum(3B)	257.50174803	14.3403

1A = Abercrombie/DeHoff and Rhines method for diameter of megakaryocytes
2A = Weibel and Gomez spheres method for diameter of megakaryocytes
3A = Weibel and Gomez ellipses method for diameter of megakaryocytes
1B = Abercrombie/DeHoff and Rhines method for of numerical density megakaryocytes
2B = Weibel and Gomez spheres method for of numerical density megakaryocytes
3B = Weibel and Gomez ellipses method for numerical density of megakaryocytes
RMSE = root mean square error
CV = coefficient of variation

Table B4. RMSE and CV of megakaryocyte quantitative methods for separated treatments

			RMSE	CV
Hypoxia	DHDia	(1A)	0.08247038	2.6592
	WGSDia	(2A)	0.11873537	3.3517
	WGEDia	(3A)	0.12121049	3.3803
	DHNum	(1B)	160.78142963	16.4849
	WGSNum	(2B)	70.13346953	9.8943
	WGENum	(3B)	70.98136220	9.5392
RAMPS	DHDia	(1A)	0.55794300	1.8061
	WGSDia	(2A)	0.04425120	1.2843
	WGEDia	(3A)	0.09515090	2.7468
	DHNum	(1B)	451.12751716	12.5368
	WGSNum	(2B)	108.51730730	3.3168
	WGENum	(3B)	113.39646150	3.4947
Control	DHDia	(1A)	0.05960436	2.2878
	WGSDia	(2A)	0.04140314	1.3564
	WGEDia	(3A)	0.04569750	1.4755
	DHNum	(1B)	601.56156352	20.4902
	WGDNum	(2B)	211.78929435	9.0659
	WGENum	(3B)	173.80464400	7.8171

1A = Abercrombie/DeHoff and Rhines method for diameter of megakaryocytes
2A = Weibel and Gomez spheres method for diameter of megakaryocytes
3A = Weibel and Gomez ellipses method for diameter of megakaryocytes
1B = Abercrombie/DeHoff and Rhines method for of numerical density megakaryocytes
2B = Weibel and Gomez spheres method for of numerical density megakaryocytes
3B = Weibel and Gomez ellipses method for numerical density of megakaryocytes
RMSE = root mean square error
CV = coefficient of variation

Table B5. Megakaryocyte diameter and number per unit volume for all treatments and quantitation methods

	Treatment (TRT)					
	1		2		3	
1A	31.00	(0.15)	30.80	(0.15)	26.00	(0.13)
2A	35.40	(0.17)	33.00	(0.16)	30.50	(0.15)
3A	35.80	(0.17)	33.70	(0.16)	30.90	(0.15)
1B	975.00	(0.04)	3598.00	(0.17)	2935.00	(0.14)
2B	708.00	(0.03)	2700.00	(0.13)	2187.00	(0.10)
3B	653.00	(0.03)	2658.00	(0.13)	2075.00	(0.10)

1A = Abercrombie/DeHoff and Rhines method for diameter of megakaryocytes
2A = Weibel and Gomez spheres method for diameter of megakaryocytes
3A = Weibel and Gomez ellipses method for diameter of megakaryocytes
1B = Abercrombie/DeHoff and Rhines method for of numerical density megakaryocytes
2B = Weibel and Gomez spheres method for of numerical density megakaryocytes
3B = Weibel and Gomez ellipses method for numerical density of megakaryocytes

TRT #1 = Hypoxia
TRT #2 = RAMPS
TRT #3 = Control
(SE)= standard error

Table B6. Megakaryocyte diameters[a] compared on smear and section preparations using the (ADH) method 1.

	Smears	Sections
Mouse		
1	40.68	25.51
2	40.57	26.10
3	39.51	25.70
4	40.20	25.30
5	40.70	25.70
6	40.80	25.20
Mean (SE)	40.41 (0.17)	25.58 (0.08)

[a]expressed in micrometers (SE)

APPENDIX C

Hematocrits. The purpose of hemocrit and red and white cell measurements was to determine and confirm the effect of hypoxia on hematopoiesis. White clay (critseal) was used to seal a drop of blood in a heparinized capillary tube. The sample of blood was centrifuged for six minutes and then read on a micro-hematocrit reader.

RBC and WBC Counts. Red and white blood cell counts were used to assess the effects of hypoxia and RAMPS treatments on erythropoiesis and granulopoiesis, respectively. Red and white blood cell counts were performed using standard methods. In the red blood cell count, blood was drawn up to the 1.0 mark using a red blood cell (RBC) pipette and then diluted with 1% ammonium oxalate to the 1.01 mark. Again the blood was diluted with isotropen 1 to 5000 and diluted again 1 to 10,000. In the white blood cell count, the blood was drawn up to 0.5 mark using a white blood cell (WBC) pipette and then diluted with 1% ammonium oxalate to the 1.0. The WBC dilutions were the same as the RBC dilutions. RBC's and platelets were lysed by hematal to obtain a pure WBC count. Both the red and white cell counts were performed on an automatic particle counter.

Platelet Counts. Platelet counts were used to assess the effect of hypoxia and RAMPS treatments on thrombopoiesis. Standard methods were followed. Blood was drawn up to the 1.0 mark using a red blood cell (RBC) pipette and then diluted with 1% ammonium oxalate to the 1.01 mark. The blood was then mixed on a pipette rotor

and put in a hemacytometer chamber under a humified cover. In the manual platelet count, the middle and the four corner squares of the hemoacytometer were counted under a phase microscope. Total platelet counts were calculated by multiplying the number of platelets counted by 5,000.

APPENDIX D

```
1     DATA ONE;
2     INPUT MSE TRT DIA NUM WGSDIA WGSNUM WGEDIA
3     WGENUM;
49    PROC SORT; BY TRT;
50    PROC GLM ; BY TRT; CLASSES BONE MSE;
51    MODEL DIA NUM WGSDIA WGSNUM WGEDIA
52    WGENUM = MSE BONE;
53    PROC GLM ; CLASSES BONE MSE TRT;
54    MODEL DIA NUM  WGSDIA WGSNUM WGEDIA
55    WGENUM = TRT MSE(TRT) BONE TRT*BONE;
56    TEST H  = TRT E = MSE(TRT);
57    ESTIMATE '1 VS 2' INTERCEPT 0 TRT 1 -1 0;
58    ESTIMATE '1 VS 3' INTERCEPT 0 TRT 1 0 -1;
59    ESTIMATE '2 VS 3' INTERCEPT 0 TRT 0 1 -1;
60    LSMEAN TRT BONE MSE(TRT)/STDERR;
61    MEANS BONE/SNK;
```

D1. The univariate computer SAS program used in analyzing diameter and number of megakaryocytes for Methods 1 (ADH), 2 (WGS), AND 3 (WGE).

```
1    OPTION LS =75
2    DATA ONE
3    INPUT EXP TRT MSE LYM NU MO EO TW;
4    NLYM= LYM*TW*.01;  NNU= NU*TW*.01;  NMO= MO*TW*.01;
5    NEO= EO*TW*.01;
6    LNLYM= LOG(NLYM+1) ;  LNNU= LOG(NNU+1);
7    LNMO= LOG(NMO+1);  LNEO= LOG(NEO+1);
35   PROC PRINT;
37   PROC GLM; CLASSES EXP TRT;
38   MODEL LNLYM  LNNU  LNMO LNEO = EXP TRT EXP*TRT;
39   MANOVA  H  TRT  EXP  E  =  TRT*EXP / SHORT;
40   MANOVA  H  EXP*TRT/ SHORT;
41   MEANS  TRT / SNK  E  = EXP*TRT;
42   PROC GLM; CLASSES EXP TRT;
43   MODEL NLYM NNU NEO = EXP TRT EXP*TRT;
44   MANOVA  H  = TRT EXP  E  = TRT*EXP / SHORT;
45   MANOVA  H  = EXP*TRT /SHORT;
46   MEANS  TRT /SNK  E  = EXP*TRT;
```

D2. The multivariate SAS computer program used in analyzing white cell numbers.

DEPENDENT VARIABLE: LNNU

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	FVALUE
MODEL	8	18.07855826	2.25981978	77.67
ERROR	18	0.52372610	0.02909589	PR> F
CORRECTED ERROR	26	18.60228436		0.0001

R-SQUARE	C.V.	ROOT MSE	LNNU MEAN
0.971846	2.1863	0.17057519	7.80196843

SOURCE	DFTYPE I SS	F VALUE	PR>F
EXP	2	0.0629461	1.08	0.3601
TRT	2	17.9185241	307.92	0.0001
EXP*TRT	4	0.9708767	0.83	0.5210

SOURCE	DFTYPE III SS	F VALUE	PR>F
EXP	2	0.0629461	1.08	0.3601
TRT	2	17.9185241	307.92	0.0001
EXP*TRT	4	0.9708767	0.83	0.5210

D3. Sample computer output using the multivariate program (SAS) used in analyzing the logarithms of the WBC differential count.

```
 10 REM "ADH/ corrected for tissue shrinkage"
 20 CLS:PRINT "Mean Diameter and N_V by Abercrombie":PRINT
 30 L$ = "ADH"
 40 INPUT "Data/", C$
 50 INPUT Experiment/Mouse Number/  ", A$
 60 INPUT "Bone/  ",O$
 70 F$ = "mm"
 80 INPUT  How many size classes/  ",C
 90 T = .003
100 INPUT  "number Feild Counted/  ",F
110 INPUT  "area/feild/  ,A
120 DIM  CD(C), CF(C), NA(C)
130 FOR I =1 TO C: READ CD(I) :NEXT I
140 DATA .0015, .0045, .0075, .0105, .0135, .0165, .0195, .0225,
     .0255, .0285, .0315, .0345, .0375, .0405, .0435, .0465,
     .0495, .0525
150 PRINT "  Class Frequencies  (smallest first) ": PRINT
160 FOR I =1 TO C: INPUT  "Class frequency  " ,CF(I) : NA(I) =
     CF(I)/(F*A): CF = CF(I) +CF: NEXT I
170 FOR I =1 TO C: SUMFCR + SUMFCR +CF(I)*CD(I): NA = NA+(I):
     NEXT I
180 D = 1.2732 * (SUMFCR/CF)
190 LPRINT "        Mean Diameter and N_V of Circular Particles
                        the Method of Abercrombie"
200 LPRINT: LPRINT
210 LPRINT  "Data              ",C$
220 LPRINT  "Program           ",L$
230 LPRINT  "Treatment/Mouse Number  ",A$
240 LPRINT  "Bone",O$
250 LPRINT  "Number of Size Classes  ", C
260 LPRINT  "Unit of Measure  ",F$
270 LPRINT  "Section Thickness  ",T
280 LPRINT  "Number of Field Counted  ",F
290 LPRINT  "Area/Field                ",A
300 LPRINT LPRINT
```

D4 (Continued)

```
310 F$ = "  ##      #.#####          ###          ###.#####
320 LPRINT   "Class#      Diameter          Freq.          Nv
330 FOR I = 1 TO C: LPRINT USING F$; I, CD(I), CF(I), NA(I): NEXT I
340 LPRINT
350 LPRINT   "Mean Profile Diameter  =, (SUMFCR/CF)
360 LPRINT  "Number Per Unit Area =  ",NA:LPRINT
370 LPRINT  "Mean Particle Diameter +  "D*1.05
380 NV = NA/(D+T) : LPRINT  "Number Per Unit Volume = ",
    NV*1.17
390 LPRINT  " ************************************
```

D4. The microcomputer program used in analyzing the mean diameter and number per unit volume for the Abercrombie method 1.

Date	08-15-87
Program	ADH
Treatment/Mouse Number	hys-3/5
Bone	ramps5/1
Number of Size Classes	15
Unit of Measure	mm
Section Thickness	.003
Number of Fields Counted	284
Area/Field	.018

Diameter Classs#	Profile Diameter	Profile Freq.	N_A
1	0.00150	3	0.58685
2	0.00450	9	1.76056
3	0.00750	14	2.73865
4	0.01050	22	4.30360
5	0.01350	28	5.47731
6	0.01650	34	6.65102
7	0.01950	65	12.71518
8	0.02250	87	17.01878
9	0.02550	76	14.86698
10	0.02850	68	13.30204
11	0.03150	45	8.80282
12	0.03450	37	7.23787
13	0.03750	15	2.93427
14	0.04050	2	0.39124
15	0.04350	1	0.19562

Mean Profile Diameter =	2.332411E-02
Number Per Unit Area =	98.98279
Mean Particle Diameter =	3.118107E-02
Number Per Unit Volume =	3541.991

D5. Sample computer output showing the calculations for mean diameter and N_V of circular particles by Abercrombie Method 1.

APPENDIX E

E1. All data used in quantitating the diameter and number of megakaryocytes.

HYPOXIA			Method 1 ADH			
		Sternum	Femur	Humerus	Tibia	Spleen
Dia	1	30.7	30.1	29.1	30.3	29.0
	2	30.1	30.8	32.1	32.7	32.2
	3	29.9	31.2	32.2	31.6	31.1
N_V	1	1150	1054	1314	1073	87
	2	1094	1513	1177	1069	107
	3	1435	1358	1024	1059	110

HYPOXIA			Method 2 WGS			
		Sternum	Femur	Humerus	Tibia	Spleen
Dia	1	3.64	33.9	34.0	34.8	33.4
	2	36.7	33.9	38.2	36.4	35.1
	3	37.2	36.3	35.3	34.3	34.8
N_V	1	791	717	866	783	75
	2	969	988	851	822	88
	3	970	881	822	911	90

HYPOXIA			Method 3 WGE			
		Sternum	Femur	Humerus	Tibia	Spleen
Dia	1	36.2	31.0	33.2	33.1	33.8
	2	35.9	32.5	37.1	34.8	35.7
	3	33.9	34.8	33.6	33.3	35.2
N_V	1	794	784	887	824	70
	2	1010	1029	876	862	82
	3	1065	920	928	939	85

E1 (Continued)

RAMPS			ADH Method 1			
		Sternum	Femur	Humerus	Tibia	Spleen
Dia	1	30.7	30.4	32.0	30.5	32.6
	2	31.1	29.4	30.6	31.0	30.6
	3	30.6	30.2	30.9	30.6	31.5
N_V	1	4422	4857	4131	3839	1244
	2	3541	5365	4025	2571	911
	3	4063	5040	4925	4070	912

RAMPS			WGS Method 2			
		Sternum	Femur	Humerus	Tibia	Spleen
Dia	1	32.2	3.48	34.0	33.7	32.1
	2	34.0	32.1	33.5	34.4	31.9
	3	33.1	30.9	33.8	33.4	31.0
N_V	1	3213	3156	3220	3116	1011
	2	2782	3623	2825	2510	789
	3	3232	3454	3622	2957	789

RAMPS			WGE Method 3			
		Sternum	Femur	Humerus	Tibia	Spleen
Dia	1	31.5	33.8	33.0	33.5	33.9
	2	33.2	30.7	33.5	33.1	32.3
	3	31.5	31.7	33.3	32.2	31.6
N_V	1	3484	3245	3323	3136	887
	2	2850	3797	2826	2610	771
	3	3401	3580	3677	3066	784

E1 (Continued)

Control			ADH method 1			
		Sternum	Femur	Humerus	Tibia	Spleen
Dia	1	25.6	25.9	25.8	25.9	26.4
	2	26.0	26.2	26.0	25.5	26.5
	3	25.9	25.8	25.9	24.6	28.1
N_V	1	3626	2895	4430	3092	1097
	2	3013	3235	2513	4284	1180
	3	2976	3372	3637	3516	1156

Control			WGS Method 2			
		Sternum	Femur	Humerus	Tibia	Spleen
Dia	1	30.7	30.0	29.9	30.3	34.1
	2	30.8	30.2	30.5	30.1	32.0
	3	30.6	29.3	29.4	30.5	32.4
N_V	1	3626	2229	2974	2166	895
	2	2174	2294	1943	2549	927
	3	2206	2552	2789	2532	945

Control			WGE Method 3			
		Sternum	Femur	Humerus	Tibia	Spleen
Dia	1	31.1	30.4	30.3	30.7	31.4
	2	31.2	30.6	30.9	30.2	32.9
	3	31.0	29.7	29.8	30.9	32.7
N_V	1	2290	2201	2935	2138	887
	2	2146	2264	1924	2539	914
	3	2177	2520	2753	2500	935

E2. Raw differential white blood cell data.

OBS	EXP	TRT	MSE	LYM	NU	MO	EO	TW
1	1	1	1	71.0	28.0	1.0	0.0	29300
2	1	1	2	73.0	24.0	1.0	1.5	26000
3	1	1	3	74.1	25.4	0.5	0.0	20000
4	1	2	4	73.5	26.0	0.0	0.5	7203
5	1	2	5	73.5	24.0	1.0	1.5	7143
6	1	2	6	75.0	24.0	1.0	0.0	6160
7	1	3	7	64.0	34.0	1.5	0.5	4034
8	1	3	8	71.5	28.0	1.0	0.0	3433
9	1	3	9	69.0	30.0	0.5	1.0	3529
10	2	1	1	67.5	31.0	1.0	0.5	29000
11	2	1	2	72.0	27.0	1.0	0.0	33100
12	2	1	3	69.5	28.5	2.0	0.0	29500
13	2	2	4	70.0	28.0	1.5	0.5	5041
14	2	2	5	67.0	31.0	1.0	1.0	7492
15	2	2	6	70.5	28.0	1.5	0.5	5053
16	2	3	7	66.0	31.0	2.0	0.5	3586
17	2	3	8	70.0	27.5	1.5	1.0	4508
18	2	3	9	68.0	30.0	1.5	0.5	3740
19	3	1	1	75.0	24.0	1.0	0.0	34000
20	3	1	2	70.0	28.0	1.5	0.5	31000
21	3	1	3	71.0	26.0	1.5	1.5	25000
22	3	2	4	70.4	25.9	2.5	2.0	5855
23	3	2	5	71.0	25.0	1.5	2.0	6120
24	3	2	6	67.0	31.5	1.0	0.5	6317
25	3	3	7	70.0	28.0	0.5	1.5	3743
26	3	3	8	67.0	29.5	1.5	1.0	4651
27	3	3	9	72.0	25.0	1.0	1.0	4605

LYM = % of lymphocytes
NU = % of neutrophils
MO = % of monocytes
EO = % of eosinophils
TW = total WBC

OBS = observation number
EXP = experiment number
TRT = treatment number
MSE = mouse number

E3. Number of each type of WBC (percent *total WBC)

OBS	EXP	TRT	MSE	NLYM	NNU	NMO	NEO
1	1	1	1	20803	8204	293	0
2	1	1	2	19110	6240	260	390
3	1	1	3	14820	5080	100	0
4	1	2	4	5294	1872	0	36
5	1	2	5	5253	1715	71	107
6	1	2	6	4620	1478	61	0
7	1	3	7	2581	1371	60	20
8	1	3	8	2454	961	34	0
9	1	3	9	2435	1058	17	35
10	2	1	1	19575	8990	290	145
11	2	1	2	23832	8937	331	0
12	2	1	3	20502	4807	590	0
13	2	2	4	3528	1411	75	25
14	2	2	5	5016	2322	74	74
15	2	2	6	3562	1414	75	25
16	2	3	7	2384	1111	71	17
17	2	3	8	3155	1239	67	45
18	2	3	9	2543	1122	56	18
19	3	1	1	25500	8160	340	0
20	3	1	2	22260	8904	477	159
21	3	1	3	17750	6500	375	375
22	3	2	4	4133	1516	146	117
23	3	2	5	4375	1530	91	122
24	3	2	6	4232	1989	63	31
25	3	3	7	2620	1048	18	56
26	3	3	8	3116	1372	69	46
27	3	3	9	3315	1151	46	46

NLYM = number of lymphocytes
NNU = number of neutrophils
NMO = number of monocytes
NEO = number of eosinophils

OBS = observation number
EXP = experiment number
TRT = treatment number
MSE = mouse number

E4. Logarithms of WBC differential counts.

OBS	EXP	TRT	MSE	LNLYUM	LNNU	LNMO	LNEO
1	1	1	1	9.9429	9.01250	5.68358	0.00000
2	1	1	2	9.8580	8.73890	5.56452	5.96871
3	1	1	3	9.6038	8.53326	4.61512	0.00000
4	1	2	4	8.5746	7.53571	0.00000	3.61132
5	1	2	5	8.5668	7.44791	4.28317	4.68403
6	1	2	6	8.4384	7.29939	4.13677	0.00000
7	1	3	7	7.8566	7.22443	4.11920	3.05259
8	1	3	8	7.8061	6.86926	3.56473	0.00000
9	1	3	9	7.7981	6.96574	2.92558	3.59154
10	2	1	1	9.8821	9.10398	5.67332	4.98361
11	2	1	2	10.0788	9.09807	5.80513	0.00000
12	2	1	3	9.9284	9.03700	6.38182	0.00000
13	2	2	4	8.1690	7.25310	4.33876	3.26595
14	2	2	5	8.5213	7.75084	4.32968	4.32968
15	2	2	6	8.1785	7.25548	4.34114	3.26824
16	2	3	7	7.7772	7.01451	4.28662	2.94075
17	2	3	8	8.0573	7.12343	4.22858	3.83038
18	2	3	9	7.8416	7.02376	4.04480	2.98062
19	3	1	1	10.1465	9.00712	5.83188	0.00000
20	3	1	2	10.0106	9.09437	6.16961	5.07517
21	3	1	3	9.7842	8.77971	5.92959	5.92959
22	3	2	4	8.3272	7.32478	4.99298	4.77153
23	3	2	5	8.3841	7.33368	4.53045	4.81543
24	3	2	6	8.3508	7.59638	4.16154	3.48385
25	3	3	7	7.8713	6.95563	2.98138	4.04559
26	3	3	8	8.0447	7.22479	4.25936	3.86094
27	3	3	9	8.1067	7.04947	3.85121	3.85121

LNLYM = logarithms of lymphocytes
LNNU = logarithms of neutrophils
LNMO = logarithms of monocytes
LNEO = logarithms of eosinophils

OBS = observation number
EXP = experiment number
TRT = treatment number
MSE = mouse number

E5. Logarithms of WBC differential counts.

OBS	EXP	TRT	MSE	LNLYUM	LNNU	LNMO	LNEO
1	1	1	1	9.9429	9.01250	5.68358	0.00000
2	1	1	2	9.8580	8.73890	5.56452	5.96871
3	1	1	3	9.6038	8.53326	4.61512	0.00000
4	1	2	4	8.5746	7.53571	0.00000	3.61132
5	1	2	5	8.5668	7.44791	4.28317	4.68403
6	1	2	6	8.4384	7.29939	4.13677	0.00000
7	1	3	7	7.8566	7.22443	4.11920	3.05259
8	1	3	8	7.8061	6.86926	3.56473	0.00000
9	1	3	9	7.7981	6.96574	2.92558	3.59154
10	2	1	1	9.8821	9.10398	5.67332	4.98361
11	2	1	2	10.0788	9.09807	5.80513	0.00000
12	2	1	3	9.9284	9.03700	6.38182	0.00000
13	2	2	4	8.1690	7.25310	4.33876	3.26595
14	2	2	5	8.5213	7.75084	4.32968	4.32968
15	2	2	6	8.1785	7.25548	4.34114	3.26824
16	2	3	7	7.7772	7.01451	4.28662	2.94075
17	2	3	8	8.0573	7.12343	4.22858	3.83038
18	2	3	9	7.8416	7.02376	4.04480	2.98062
19	3	1	1	10.1465	9.00712	5.83188	0.00000
20	3	1	2	10.0106	9.09437	6.16961	5.07517
21	3	1	3	9.7842	8.77971	5.92959	5.92959
22	3	2	4	8.3272	7.32478	4.99298	4.77153
23	3	2	5	8.3841	7.33368	4.53045	4.81543
24	3	2	6	8.3508	7.59638	4.16154	3.48385
25	3	3	7	7.8713	6.95563	2.98138	4.04559
26	3	3	8	8.0447	7.22479	4.25936	3.86094
27	3	3	9	8.1067	7.04947	3.85121	3.85121

LNLYM = logarithms of lymphocytes
LNNU = logarithms of neutrophils
LNMO = logarithms of monocytes
LNEO = logarithms of eosinophils

OBS = observation number
EXP = experiment number
TRT = treatment number
MSE = mouse number

VITA

Richard Frank Pressley was born in Knoxville, Tennessee on October 24, 1962. He attended elementary school in that city and was graduated from Fulton High School in June 1981. He entered the University of Tennessee in July 1981, and graduated in June of 1985 with a degree in Zoology. He entered the Master's program and then accepted a research assistantship the following summer of 1986. In August of 1988, this degree was awarded with a major in Comparative and Experimental Medicine.