FUNDAMENTALS OF TOXICOLOGIC PATHOLOGY

Editors:

Wanda M. Haschek-Hock
University of Illinois

Colin G. Rousseaux
University of Saskatchewan

CHAPTER 16

BLOOD AND BONE MARROW

RICHARD D. IRONS, M.T., Ph.D.

Molecular Toxicology and Environmental Health Sciences Program

School of Pharmacy and Department of Pathology

School of Medicine

University of Colorado Health Sciences Center, C-235

4200 E. 9th Avenue

Denver, Colorado 80262

ACADEMIC PRESS, IN **PRESS**

Final October 25, 1989

BLOOD AND BONE MARROW

RICHARD D. IRONS, M.T., Ph.D

Molecular Toxicology and Environmental Health Sciences Program
School of Pharmacy and Dept. of Pathology
School of Medicine
Univ. of Colorado Health Sciences Center, C-2.38
4200 E. 9th Ave.
Denver, Colorado 80262

BLOOD AND BONE MARROW

Richard D. Irons

Table of Contents

#1 HD Introduction

#2 HD Blood Cells

The cells of the blood and bone marrow constitute a widely dispersed and highly regulated organ system, that provides a number of critical functions for the host. Erythrocytes provide a means to transport and maintain hemoglobin for the delivery of oxygen, and act as a buffer to facilitate the removal of carbon dioxide. Granulocytes serve as a nonspecific, or constitutive, host defense against foreign organisms, functioning both as phagocytes and as mediators of inflammation. Lymphocytes serve as specific mediators of immune response; final arbiters in the recognition of foreign antigens. In addition, certain subpopulations apparently play a role in the regulation of hematopoiesis. Macrophages can be considered together with their circulating precursors, monocytes. They fulfil an intermediate role between constitutive host resistance and immune response, by functioning either as end-stage effector cells in phagocytosis and/or cell killing, and/or as regulatory cells in hematopoiesis and the immune response. Finally, platelets play an important role in hemostasis and in maintaining vascular integrity.

When considering target organ toxicity, individual cell lines are often considered separate systems; however, all cell lines share a common origin (the pluripotent stem cell of the bone marrow), are interrelated, and are coordinated during hematopoiesis. Rarely is there a significant adverse effect solely on one cell line.

Experimental hematology has witnessed 1ogarithmic growth, and an increase in sophistication over the last few years. This growth has been brought by advances in cell and molecular biology. This chapter will attempt to discuss toxicologic pathology of the blood and bone marrow in the context of our growing, but incomplete, knowledge of the molecular and cellular events that regulate hematopoiesis.

#1 HD Structure and Cell Biology

#2 HD Gross and Microscopic Anatomy

#3 HD Mass and distribution of hematopoietic tissue

In the normal adult, hematopoiesis is largely confined to the medullary cavity of bones. In man, dog and rabbit, hematopoietic tissue occupies all medullary space at birth, but becomes progressively limited to the proximal epiphyses of the long bones, central skeleton and skull as the individual ages. Even so, adult bone marrow constitutes 1 - 5% of the body mass, depending on the species. In contrast, essentially all medullary space is occupied by functioning hematopoietic tissue throughout 1ife in mice, and rats.

Increased hematopoietic activity in adult humans is confined to bone medullary spaces, whereas extramedullary hematopoiesis in the spleen, liver and lymph nodes occurs only under conditions of extreme demand. In fact, hematopoiesis is restricted to the bone marrow lumen even under the radical

conditions of bone marrow transplantation, following total bone marrow destruction by whole body irradiation. In contrast, isolated islets of erythropoietic cells are normally found in the spleen of the rat, and occasionally small clusters can be found in the liver. In the mouse and rat, the spleen stands poised to become a major hematopoietic organ under the influence of increased demand.

The unique predisposition of the spleen to serve as a hematopoietic organ in the rodent is an important consideration in the use of mice and rats as animal models. Compensatory splenic erythroid, lymphoid, or myeloid hyperplasia may confounding the differential diagnosis of splenic myeloproliferative disease, lymphoma or other neoplasia. To add further confusion, most kinetic parameters used to assess the effect of drug, or chemical exposure, on hematopoietic cell proliferation assume the mass of hematopoietic tissue remains constant. Therefore, the relative number of lymphocytes or macrophages, in addition to their contribution to the overall proliferating splenic cell population may vary considerably depending on the degree of splenic extramedullary hematopoiesis encountered. These sources of variation are particularly important in the assessment of the effect splenic hematopoietic effects in <u>in vitro</u>, as most assays of immune function in the rodent rely on the spleen as a convenient source of lymphocytes or macrophages.

#3 HD **Microscopic** Anatomy

Bone marrow contains reticular cells that form a three dimensional latticework in which hematopoiesis occurs. Anatomically and functionally, it

can be divided into vascular and extravascular compartments. Blood enters the marrow via the nutrient artery, which bifurcates to form the central artery of the medullary canal. Radial or medullary arteries then branch from the central artery forming arterioles, some of which terminate in capillaries within the medullary space, while others penetrate the endosteum. These capillaries supply blood which mixes with blood from the periosteum. Blood returns by means of the vascular sinuses to the central vein.

Hematopoiesis takes place within the convoluted extravascular spaces of the bone marrow sinuses. These sinuses are lined by a single layer of endothelial cells which interdigitate with adventitial, reticular, or dendritic cells. Their fibres form the numerous compartments of the extravascular space and support cords of hematopoietic cells. These stromal cells, which are adherent in culture, play a major role in the regulation of hematopoiesis. They form the niches in which hematopoiesis takes place, retract to facilitate transmural migration of blood cells through the endothelial lumen. Cultured bone marrow stromal cells have been shown to support hematopoiesis in vitro, and to produce a variety of growth factors essential for the support and regulation of proliferation and differentiation of hematopoietic cells. Trentin [1] introduced the concept of the "hematopoietic inductive microenvironment" to describe the influence of these stromal cells on stem cell function and the proliferation of hematopoietic precursor cells.

---

[1] Trentin, 1971

Although there is no evidence for any specific organization or localization of precursor cells within the extravascular cords of the bone marrow, the distribution of cells within individual niches does not occur at random. Clusters of differentiating cells of a given lineage are often seen within an individual niche. For example, erythroblasts are often observed forming rosettes around a centrally placed dendritic or macrophage-like cell; megakaryocytes are frequently found adjacent to bone marrow sinuses, which undoubtedly facilitates the release of platelets directly through the sinus wall.

There are no major species differences in the architecture of the bone marrow. As previously mentioned, the entire medullary space of mice and rats is occupied by hematopoietic tissue throughout life; therefore, the bone marrow of these species contains little or no fat. In contrast, adult bone marrow in man, dog and rabbit contains many adipose cells. Bone marrow of the rat can be distinguished from mouse and man by larger numbers of mast cells with prominent basophilic granules. In fact, careful preparation of bone marrow smears from the rat is important to minimize the disruption of mast cells whose granules can obscure other cells.

#2 HD Hematopoiesis

In the normal steady state, senescent blood cells are continually replaced by cells originating in the bone marrow. This replacement amounts to 2-3 X 10" of cells/day for an average human adult. Hematopoiesis is maintained and regulated through a process of amplification and differentiation, in which immature progenitor cells give rise to a larger

number of progressively more differentiated generations of cells (Figure 1).

The hematopoietic cells of the bone marrow are divided into four compartments, based of their tendency to differentiate and their proliferative capacity. At the apex of this pyramid is the **pluripotent stem** cell **(PSC)**, which is capable of giving rise to progenitor cells in ***any*** lineage, but appears to have a very limited proliferative capacity even though it has unlimited self-renewal characteristics. With each division in progressive differentiation of the PSC an associated increase in proliferative capacity and a corresponding decrease in self-renewal potential occurs. Lineage-committed or mortal progenitor cells, have a more limited repertoire of gene expression, but an increased tendency to proliferate enabling them to produce precursor cells in 1-3 of the hematopoietic lineages. The morphologically recognizable and highly proliferative precursors of the various cell lineages give rise to the end-stage cells of the blood. With the exception of lymphocytes and monocytes, end-stage blood cells have no proliferative capacity.

#3 HD Stem **and progenitor** cells

A tribute to the insight of hematologists early in this century is the observation that all blood cells were descendants of a common stem cell as early as the 1930's. Not until 30 years later was the clonal nature of hematopoiesis experimentally confirmed. Pluripotent stem cells **(PSC)** represent an exceedingly small compartment of cells in bone marrow (1 in 10^3 - 10^5 nucleated cells, depending on the species). Historically, direct studies on the behaviour and regulation of stem cell populations were, and still are,

hampered by the lack of reliable morphologic criteria. Despite success in **PSC** identification and purification, stem and progenitor cell populations continue to be defined functionally by clonal assays, because of their scarcity.

#4 HD **COLONY-FORMING UNIT-SPLEEN** (CFU-S)

In reconstitution experiments with irradiated mice, Till and McCulloch observed that certain bone marrow cells had the ability to form hematopoietic colonies in the spleens of lethally irradiated syngeneic recipient mice. They observed that this event coincided with the capacity for self renewal and the ability to produce cells of the myeloid, erythroid, and megakaryocyte lineages. Since then, the colony-forming unit-spleen (CFU-S) assay has been widely adopted as a quantitative assay for the estimation of PSC in mouse bone marrow. Subsequent studies demonstrated that a subset of cells within the **CFU-S** are capable of giving rise to all hematopoietic and lymphoid lineages.

The CFU-S pool is now recognized to consist of a heterogeneous group of cells, remarkably variable in their individual capacities for proliferation, differentiation and self-renewal. Two subsets of **CFU-S** can be distinguished on the basis of their propensity for self-renewal and differentiation: **CFU-S I**, which have greater capacity for self-renewal and proliferation and are non-cycling, and CFU-S II which have much less self-renewal and proliferative capacity but cycle actively.

New culture techniques for stem cell populations in semisolid media have revealed overlapping cell populations, with characteristics intermediate between **PSC** and committed stem cell populations. The CFU-Mix cell, for

example, is capable of giving rise to myelocytic, erythrocytic and potentially lymphocytic cell lineages, yet apparently has limited capabilities for self-renewal. At present, it is thought the CFU-S II and CFU-Mix are the same cell populations detected by different assay procedures.

To date, equivalent assays for CFU-S have only been characterized in mice and rats, although there is evidence that a similar cell may colonize the spleen in dogs following radiation. However, a number of <u>in vitro</u> culture systems have been developed that permit the enumeration of committed or progenitor cell lines in a variety of species.

#4 HD Progenitor Cells

Characterization of early committed progenitor cells followed studies in the mid 1960's which showed colonies of individual progenitor cells could be grown in culture supported by conditioned medium. Subsequently, a variety of growth factors or colony stimulating factors (CSF's) have been identified that support the growth of specific hematopoietic precursor cells <u>in vitro</u>. The most primitive erythroid progenitor cell is the burst forming unit-erythroid (BFU-E), which gives rise to large colonies of erythroid precursor cells which appear after 6 days in semisolid culture in the presence of high concentrations of erythropoietin (Epo). BFU-E is now thought to represent a very early cell in the commitment process, that is responsive to IL-3 (see below). The cell directly responsive to Epo is the colony forming unit-erythroid (CFU-E) which gives rise to smaller colonies of erythroid cells within 2-3 days of culture on semisolid media.

Colonies consisting of predominantly granulocytes, macrophages, or a mixture of both, can also be obtained from a culture of bone marrow cells in semisolid media depending on the source of humoral stimulation used. These cells have been classified as colony forming unit-granulocyte-macrophage (CFU-GM) and colony forming unit macrophage (CFU-M). Additional colonies have been identified in culture for various lineages including: megakaryocytes (CFU-Meg), fibroblasts (CFu-F) and pre-B lymphocytes (CFU-BL). There is also limited evidence to demonstrate the existence of a pre-T lymphocyte population in bone marrow.

#3 HD Regulation

#4 HD The Microenvironment

The microenvironment plays a critical role in maintaining the immortality of the PSC, and in regulating progenitor cell activity. That hematopoiesis could be subject to regulation by soluble factors was recognized at the turn of the century when serum from anemic rabbits was shown to increase RBC in normal animals. In addition, there is also evidence to indicate contact with stromal cells is necessary to support and regulate stem cell differentiation.

The dual importance of stem cell and stromal cell components in the maintenance of normal hematopoiesis is illustrated by genetic anemia in mice. Sl/Sl^d and W/W^v mice both exhibit macrocytic normochromic anemia; however, the pathogenesis of the anemia differs. W/W^v mice possess a congenital stem cell defect that is correctable by transplantation with normal bone marrow cells,

whereas transplantation of stromal cells is necessary to correct the anemia in Sl/Sl^{d} mice. In addition to providing a matrix for hematopoietic cells, the stromal fibroblasts produce soluble products that regulate hematopoiesis. There is evidence macrophages may regulate stromal secretions through IL-1 production.

T-lymphocytes also participate in the regulation of hematopoiesis. Selective removal of T-lymphocytes from erythroid progenitor cell populations reduces BFU-E in vitro. T-cell depleted bone marrow will not cure anemia in the W/W^{v} mouse. In addition, pre-sensitized T-lymphocytes produce an antigen-specific increase in eosinophil production in vitro.

#4 HD **Growth** Factors

The regulation of hematopoiesis involves a complex set of interactions among growth factors and differentiating cells, mediated via surface receptors on target cells. Expression of these receptors is linked to differentiation of hematopoietic precursor cells. While cell differentiation may be a stochastic process, it appears to be dependent on the availability of various growth factors. A growth factor may act directly on a progenitor cell, or indirectly via interaction with accessory cells to alternatively stimulate or suppress hematopoiesis; hematopoiesis requires a fine balance between stimulatory and inhibitory factors.

Although more detailed knowledge of the number, role and control of growth factors and receptor expression will be forthcoming, a rudimentary understanding of these factors is available (Table 1). Several growth factors

have been molecularly cloned and purified, and are the subject of intensive laboratory and clinical investigation.

Interleukin 3 (IL-3), originally coined "Multi-CSF" and subsequently renamed, is a product of T-lymphocytes that serves as a multi-lineage growth or viability factor. It acts at an early stage in hematopoietic cell differentiation, permitting the growth and survival of PSC. In addition, IL-3 supports growth and differentiation of early restricted lineage or progenitor cells, such as granulocyte/macrophage, erythroid, megakaryocyte, mast cell and pre-lymphocyte lineages.

Two growth factors appear necessary to support the development of erythroid cells: IL-3 and Epo. IL-3 supports the development of PSC and lineage committed progenitor cells including early erythroid progenitors (BFU-E). A complex glycoprotein produced mainly in the kidney, Epo stimulates the development of CFU-E, which appears to be derived from proerythroblasts or their immediate precursors.

A variety of colony stimulating factors (CSF), including macrophage colony stimulating factor (M-CSF), granulocyte/macrophage colony stimulating factor (GM-CSF), and granulocyte colony stimulating factor (G-CSF) are all produced by bone marrow fibroblasts and endothelial cells. Monocytes produce M-CSF, and T lymphocytes produce GM-CSF and M-CSF. When studied singly, M-CSF, GM-CSF and G-CSF predominantly stimulate the formation of macrophage, macrophage or granulocyte and granulocyte colonies, respectively.

Concentration-dependent interactions between various growth factors are complex. For example, concentrations of GM-CSF stimulate primarily the formation of granulocyte colonies, whereas low concentrations result in formation of macrophage colonies. GM-CSF has also been demonstrated to support megakaryocyte and erythroid colony formation. In the presence of G-CSF, granulocyte colonies predominate at low concentrations; macrophage colonies are observed only at high concentrations. IL-3 can potentiate the production of progenitor colonies in the presence of various committed growth factors. Synergistic effects following coadministration of IL-3, M-CSF and GM-CSF have been observed in mice both *in vivo* and *in vitro*.

In contrast to growth factors, a number of inhibitory molecules have effects on hematopoiesis, including: lactoferrin (LF), prostaglandins (PGE), the interferons (XFN) and tumor necrosis factor (TNF). LF is a metal-binding glycoprotein produced by granulocytes which suppresses the release of GM-CSF and other stimulatory factors. Monocytes and macrophages express surface receptors specific for LF, and some evidence suggests that LF multi-lineage suppression is mediated through suppression of IL-1 production by macrophages. Type E prostaglandins (PGE) exert a complex influence over hematopoiesis by selectively inhibiting macrophage precursor cells and stimulating CFU-GM. PGE, makes cells of multiple lineages responsive to feedback inhibition by the induction of Ia surface antigen expression; it also stimulates the release of Epo by the kidney and stimulates erythroid colony formation *in vitro*. Interferons are a family of glycoproteins, originally studied for their antiviral activity, that suppress hematopoietic progenitor cells. IFN-gamma has been implicated in the pathogenesis of aplastic anemia and, in conjunction with IFN-alpha, synergistically suppresses CFU-mix, BFU-E and CFU-GM. Tumor

Necrosis Factor (TNF), from macrophages and natural killer cells, has a cytolytic effect on certain tumor cells, and stimulates the release of IFN and IL-1.

In addition to stimulation of progenitor cells, many CSF's have effects on mature end-stage cells. For example, G-CSF and GM-CSF are chemotactic for migrating neutrophils, and stimulate phagocytosis. Thus, in response to bacterial infection, not only do CSF's result in the increased production of the responding cells, but they also prime these cells to meet the specific challenge. Thus, there is considerable evidence to suggest that the cells of the hematopoietic and immune systems collaborate in an elaborate network to regulate and direct hematopoiesis.

The use of in vitro studies to evaluate the effects of chemical or drug treatment on the elaboration of individual growth factors holds great promise as an approach to elucidate molecular mechanisms of toxicity to the hematopoietic system. Nevertheless, several important questions remain to be answered before the results of individual studies can be interpreted with confidence.

Despite the influence of the microenvironment and individual growth factors on PSC survival or the growth of lineage-specific progenitor cells, PSC differentiation and proliferation are a random processes. Haw microenvironment and growth factors interact with humoral regulation of lineage-specific progenitor cells is not entirely understood at present, nor is our knowledge concerning the effect of individual growth factors on hematopoiesis in vivo complete. For example, do fibroblasts produce

hematopoietic growth factors *in vivo*? If all fibroblasts (e.g. lung, peritoneum, bone marrow etc.) can produce growth factors, what restricts or regulates this function in specific tissues? By now it should be apparent how complex are interactions among CSFs; demonstrating an effect on the elaboration of a single growth factor is unlikely to reflect intricacies of the toxic response *in vivo*.

#4 HD Clonal succession

The role of clonal succession in the regulation of hematopoiesis is of considerable consequence in understanding potential mechanisms of leukemogenesis. The theory of clonal succession was proposed a potential protective or regulatory mechanism for hematopoiesis, in which rapidly proliferating hematopoietic precursor populations possess a restricted capacity for independent proliferation. For such populations, terminal differentiation is linked to proliferation. The effect of this linkage is to decrease the effect of genetic or nondisjunctional errors in rapidly proliferating precursor cells; therefore, control of PSCs diminishes the opportunity for incorporation of errors into an immortal cell line. The alternative hypothesis states PSC regulation *in vivo* is *entirely* a stochastic process, and multiple PSCs are activated at any one time.

Cloning and growth of individual progenitor cells has greatly facilitated elucidation of PSC regulation; however, controversy still exists concerning the mechanisms that govern PSC proliferation and differentiation. How many PSC are actually responsible for populating the hematopoietic system at any one time? The alternatives are many, or very few. It is known that

the entire hematopoietic system can be reestablished by very few, perhaps only a single PSC, capable of giving rise to multiple lineages of cells, a process demonstrated in vitro. However, the question of stem cell activation has only recently been approached directly in vivo.

One approach to address this question has utilized retroviral-mediated gene transfer techniques, which permit high efficiency labelling of individual stem cells, thereby providing a direct means to follow the activation of individual stem cells and their progeny. Recent experiments, utilizing vector mediated labelling of hematopoietic stem cells in reconstituted mice, indicate 1 or 2 stem cell clones can account for the majority of hematopoietic cells in the body at any one time. Changes occur in the relative contribution of various clones with time. These results suggest there is a sequential utilization of PSCs. They imply an in vivo mechanism for temporal control of stem cell activation exists. In contrast, by purifying of PSC populations, reconstitution of mice with increasing numbers of PSCs leads to an increased number of clones contributing to spleen or thymic colonies. Other researchers have concluded, based on estimates derived from the variances in individual mutational events in mature erythrocyte populations, multiple PSC activation is the rule. Resolution of this debate is likely to impact heavily on the future design and interpretation of bone marrow toxicity studies.

#2 HD Cells of the Blood

#3 HD Erythrocytes

#4 HD Morpho1ogy and Function

The mature erythrocyte in mammals is an anucleate biconcave disc. This shape maximizes the surface area to volume ratio, critical for optimal gas exchange, and is readily deformable as the cells pass through capillaries and the reticular network of the spleen. The normal erythrocyte can pass through apertures less than half its own diameter. Deviations in the shape of the erythrocyte are called poikylocytosis. Such deviations reduce efficiency of oxygen transport, and can occur following direct cell membrane damage or osmotic shock. Poikylocytosis is also seen in disorders of erythropoiesis, such as megaloblastic or hypoplastic anemia. Increased erythrocyte rigidity accompanies poikylocytosis may contribute to reduced erythrocyte 1ifespan.

The size of the erythrocyte is subject to considerable species variation (diameters range 3 μm in goats to 8μm in humans). Abnormal variation in erythrocyte size, termed anisocytosis, is usually the result of response to erythrocyte loss or injury. Accelerated release of reticulocytes (immature RBC) in response to RBC loss will result in an increase in the mean corpuscular volume (MCV) of erythrocytes, or macrocytosis, because reticulocytes are 1arger than mature RBC. Abnormalities in precursor cell maturation associated with drug-induced dysplasias or megaloblastic anemia will also result in an increase in the MCV. These anemias are usually accompanied by megaloblastic changes in precursor cells in bone marrow or spleen. They can be distinguished from hemolytic anemias by the absence of reticulocytosis.

Several different terminologies have been applied to the identifiable cell populations in erythroid maturation, only two of which will be employed in this chapter. The reader is directed to current hematology texts for a more thorough discussion of the subject. The first recognizable erythroid precursor cell in the bone marrow is the proerythroblast or rubriblast. In Romanowsky-type stained preparations, the nucleus of the proerythroblast has pale staining chromatin network containing one or two nucleoli. The cytoplasm is blue without granules. The basophilic erythroblast or prorubricyte also has pale-staining nuclei, but has darker blue cytoplasm. As the basophilic erythroblast matures the chromatin of the nucleus (rubricyte) becomes progressively more condensed. The appearance of a purple or "polychromatic" hue to the cytoplasm marks the transition to active hemoglobin synthesis in polychromatophilic erythroblasts (rubricytes). The nucleus in these cells further condenses becoming pyknotic, at which stage the orthochromoblast or metarubricyte (nucleated erythrocyte) is formed. Extrusion of the nucleus gives rise to the immature erythrocyte or reticulocyte, which still contains ribosomal subunits that maintain active hemoglobin synthesis for approximately 2 days after release into the circulation. Erythropoiesis is one of the most impressive examples of differentiation in nature.

#4 HD Production and Fate.

The lifespan of the circulating erythrocyte varies considerably from one species to another. Unlike granulocytes and lymphocytes, the erythrocyte spends its entire lifespan confined to the circulation. The average erythrocyte lifespan in humans is approximately 120 days compared to about 30

days in the mouse. The spleen plays a major role in the sequestration and removal of damaged RBC, although secondarily, other reticular tissues fulfil this function. Daily turnover of over 10^{11} RBC occurs in man compared to 10^{9} RBC in the mouse.

#3 HD Granulocytes

#4 HD Morphology

The granulocytic series consists of three cell lines that share common developmental and morpho1ogic features. Mature granulocytes are cells of 10-20 μm diameter characterized by a segmented nucleus are termed "polymorphonuclear" leukocytes (PMNs). They contain azurophilic and specific cytoplasmic granules, distinguishable by 1ight microscopy. Classification of granulocytes into neutrophils, eosinophils and basophils is based on the staining characteristics of their primary cytoplasmic granules in Romanowsky-stained smear preparations.

Azurophilic granules are found in all three granulocytic cell types. In mammals, the predominant granulocyte is the neutrophil which has 3-7 lobed nucleus and specific granules that usually stain a beige color. A species difference can be seen in the heterophil or "pseudoeosinophil" of the rabbit and guinea pig, where the specific granules stain an intense orange pink to red. Eosinophils usually contain 2-3 lobed nuclei and red specific granules, which vary in size and shape among species. The specific granules of basophils stain intensely basophilic and vary dramatically in size from one species to another.

All three granulocytic cell types exhibit comparable stages in development and differentiation. The first recognizable precursor of the granulocyte lineage is the myeloblast. Its nucleus has a delicate pale staining chromatin network, and usually contains three or more large nucleoli. Myeloblast cytoplasm is pale blue containing a few azurophilic granules, the distinguishing feature of this cell. The increase in the number of azurophilic granules in the cytoplasm characterizes the promyelocyte. The nucleus of the promyelocyte is frequently kidney shaped and may contain remnants of the once prominent nucleoli. Its cytoplasm varies from light blue to the pale pink of the mature granulocyte. As the promyelocyte matures, the chromatin of the nucleus condenses and a second type of granule, the specific granule, appears. An increase in the number of specific granules, together with the presence of a pale pink cytoplasm, are the hallmarks of the myelocyte. As the myelocyte continues to differentiate, the nucleus further condenses to form a rod or "band" appearance. Further nuclear condensation results in the multi-lobulated appearance of the mature granulocyte. Nuclei of granulocytic progenitor cells and mature granulocytes in some species often contains a centrally placed vacuole. These "donut" cells are normally found in rodent bone marrow, and become more prevalent upon stimulation of granulopoiesis. In humans, their presence is associated with severe pathology, such as that encountered in myelodysplastic syndrome or leukemia.

#4 HD Function

The cytoplasmic granules of granulocytes are lysosomes which concentrate a variety of enzymes and biologically active substances used in phagocytosis,

killing and inflammation. The contents of azurophilic granules include: myeloperoxidase, acid phosphatase, esterase, beta-glucuronidase and lysosyme. Neutrophilic specific granules contain alkaline phosphatase lysosyme and lactoferrin. Eosinophilic granules are rich in plasminogen as well as a variety of hydrolytic enzymes including: acid phosphatase, glucuronidase, phospholipase, cathepsins and ribonuclease. Although eosinophilic granules exhibit peroxidase activity, this activity is different to myeloperoxidase of neutrophils. Basophilic granules contain, in addition to a variety of enzymes, heparin and histamine.

The primary function of granulocytes is bacterial phagocytosis and killing. Secondary functions include the release of pyrogens and lactoferrin, the latter serving in a feedback loop to control granulopoiesis. Certain cellular functions are critical to the role of granulocytes in the circulation. Among these are margination or adherence to endothelial cells; chemotaxis, i.e., diapedesis through the vessel wall and migration toward a chemotactic stimulus; attachment and phagocytosis of bacteria; fusion of lysosomes with phagocytic vacuoles, subsequent degranulation and killing.

Adherence involves the secretion of lactoferrin and a complement receptor. It is enhanced by complement activation or leukotriene B4 (LTB4).

Chemotaxis is stimulated by a variety of substances including bacterial products such as endotoxin, activated complement and LTB4. Chemotaxis is dependent on signal transduction through the plasma membrane (including calcium flux), and cytoskeletal integrity.

Phagocytosis requires recognition of, and attachment to, a foreign particle. This process is facilitated by opsonization, where the foreign

particle is coated by immunoglobulins. Following engulfment of the particle, the internalized vacuole undergoes fusion with a lysosome. Lysosomal degranulation leads to the activation of hydrolytic enzymes, and the induction of the leukocyte oxidative burst, which mediate bacterial killing.

In addition to these generalized functions of PMN's, eosinophils are attracted by mediators of allergy, such as histamine and serotonin. Basophils are the circulating equivalent of tissue mast cells, and play a role in immediate hypersensitivity and inflammation. In species such as the rat, which have a relatively high number of mast cells in the bone marrow and reticular tissues, circulating basophils are exceedingly rare.

#4 HD Production and Fate

Unlike the erythrocyte, which spends its entire lifespan confined within the circulatory system, the time the granulocyte spends in the circulation is extremely short, i.e., about 10 - 14 hr. Granulocytes leave the blood vessels by passing between endothelial cells in a process known as diapedesis. After diapedesis, the lifespan of the granulocyte in peripheral tissues is thought to be 2-3 days. A major route of elimination of granulocytes involves transmural migration through the gut wall. Because transit time is short, and lifespan in peripheral tissues of the granulocytes is only days, decreased production or increased destruction of granulocytes rapidly effects the circulating pool. As a result, granulocytopenia is frequently an early finding in bone marrow suppression.

[Insert Figure 2 here]

The granulocyte pool is unevenly distributed between the maturation and storage compartment of the bone marrow (90%) and the circulation (10%) (Figure 2). Granulocytes in the peripheral blood are equilibrated between two compartments: circulating and marginated. Circulating granulocytes comprise that population accessible during routine blood counts. An equal number of cells are marginated, or adhered to the endothelial wall, and can rapidly released into the circulating pool. Since the peripheral blood granulocytes (both circulating and marginated) constitute only about 10% of the total granulocyte maturation/storage pool, the release of only a very small proportion of storage/maturation compartment can result in a doubling of the peripheral granulocyte count. Glucocorticoids, for example, produce granulocytosis, due to release of cells from the bone marrow compartment, decreased adherence and decreased egress.

#3 HD Monocyte-Macrophages

#4 HD Morphology

Macrophages comprise an array of tissue-specific and circulating cells derived from bone marrow precursor cells and the circulating monocyte. These cells include: alveolar macrophages, the **Kupffer** cells of the liver, tissue histiocytes, splenic, lymph nodes, and bone marrow macrophages. The typical circulating monocyte is a large cell, about 30 microns in diameter, containing a large irregularly shaped nucleus. In Wright's or Giemsa-stained preparations, the nuclear chromatin is delicately filamentous. Its abundant cytoplasm, is sky blue or grey and may contain numerous fine azurophilic granules and vacuoles. Although a blast cell with characteristics resembling

monocytes is frequently observed in bone marrow preparations and is defined as a "monoblast", a morphologically identifiable maturation sequence of monocyte precursors in the bone marrow has not been identified.

#4 HD Function

Macrophages are phagocytes, and function to remove foreign bodies, parasites and bacteria; however, significant tissue specific differences in individual macrophage phagocytic functions exist. For example, intravenous carbon particles given to rats are more efficiently removed by Kupffer cells than by splenic macrophages, whereas the opposite is true for foreign or damaged RBC.

The fate of administered antigens varies markedly from one species to another. Intravenously administered particulate antigens accumulate primarily in the liver of rats, whereas the lung is the primary site for deposition of blood borne particulates in sheep and goats. Mice and rats clear inhaled particulates much more rapidly from the lung than dogs. Differences in the fate of antigens and their pattern of distribution may play a significant role in explaining major species differences in immune response and disease patterns. Bone marrow macrophages are now thought to play a supportive role in hematopoiesis through the secretion of IL-1 which in turn stimulates the release of CSF's from fibroblasts, T-lymphocytes and endothelial cells. Finally, macrophages play a major role in antigen presentation and regulation of immune response.

#4 HD Production and **Fate**

Monocytes have an intravascular lifespan of 1-3 days. They leave the blood stream at random. There does not appear to be any significant return traffic once monocytes have left the circulation. Although the transformation of monocytes to macrophages has been long appreciated, whether these cells can normally undergo further proliferation after leaving the bone marrow is still under debate. The lifespan of macrophages in tissues is on the order of months.

#3 HD Platelets

#4 HD Morphology

Platelets are granular cytoplasmic fragments that take on a variety of shapes depending on their state of activation and the quality of the smear. Their size is also variable, depending on the preparation and the species. They have a marked tendency to agglutinate and often form large aggregates on peripheral blood smears. Platelets are generally round, oval or tear drop in shape, measuring between 1 and 5 microns in diameter. Despite their amorphous appearance, platelets are highly organized structures.

The surface of the platelet is rich in glycoproteins and acid mucopolysaccharides. Their cytoplasm contains numerous microfilaments, is supported by a network of microtubules, and is slightly basophilic containing purple granules. Platelet granules contain a variety of factors that accelerate clotting, promote clot contraction, and growth factors which promote wound healing. These include: Serotonin, epinephrine, fibrinogen and

platelet derived growth factor. Enzymes of the cyclooxygenase pathway are also present for the synthesis of prostaglandins and thromboxanes.

Megakaryoblasts are the earliest recognizable precursor cells of the thrombocytic lineage, and are extremely large cells, measuring up to 100 μm in diameter. They are distinguished from other hematopoietic precursor cells in that they are polyploid. They undergo a sequential maturation to megakaryocytes, involving nuclear segmentation and the acquisition of cytoplasmic granules before fragmenting to form platelets. During platelet formation, megakaryocytes extend filamentous cytoplasmic projections through the endothelial wall which break off releasing platelets directly in the circulation.

#4 HD Function

Platelets play a major role in hemostasis through the initiation of clot formation. They are activated by contact with collagen, air or foreign surfaces. Activation (usually via adhesion) brings about the release of multiple factors causing aggregation, increased adherence to endothelial surfaces and the release of clotting factors III and IV. Platelets also release growth factors, such as platelet-derived growth factor (PDGF), thought to promote tissue regeneration following injury.

#1 HD Xenobiotic Exposure and Metabolism

Hematotoxic compounds can be divided into two major categories: pharmaceuticals and occupational hazards. Exposure routes used in metabolic and toxicity studies include parenteral, oral or inhalation routes. The influence of xenobiotic biotransformation and pharmacokinetics on bone marrow toxicity is a complex; many compounds that produce bone marrow toxicity require bioactivation, but exceptions exist. Major exceptions appear to be reactive antineoplastic agents, such as hydroxyurea, 5-azacytidine, the nitrogen mustards, and antibiotics such as puromycin.

For many myelotoxic agents, metabolic studies are complicated by their chemical reactivity. For example, studies on the pharmacokinetics and fate of the nitrosoureas have been hampered by their rapid degradation. Such difficulties are often further compounded by unequal distribution indifferent tissues, a fact highlighted by studies on the nitrosoureas and most recently 1,3-butadiene. Furthermore, detailed comparative studies of species differences in xenobiotic metabolism, pharmacokinetics and disposition, have rarely been performed simultaneously with experiments designed to measure bone marrow toxicity. As a result, it is often difficult to interpret the precise role of secondary and tertiary metabolism in myelotoxicity.

The reactions associated with the metabolism of hematotoxic agents are the same as for other xenobiotics; they involve primarily oxidation, hydroxylation, demethylation, deamination, conjugation and nitroreduction. Not surprisingly, the liver is the principal site of primary oxidative

metabolism; however, in certain situations there is evidence that bone marrow metabolism plays a major role in conferring susceptibility. For example, there is evidence that tertiary metabolism of the hydroxy-metabolites of benzene in the bone marrow play a major role in target organ toxicity [2]. Although increasing attention is being given to the role of bone marrow in the bioactivation of foreign compounds and metabolites, the role if any of tissue specific detoxification in hematotoxicity is unknown.

#2 HD Metabolism of Hematotoxic Compounds

Cyclophosphamide is a classical example of a compound that has no intrinsic cytotoxicity but requires bioactivation via the cytochrome P-450 mixed function oxidase system to produce the alkylating secondary metabolites, phosphoamide mustard and acrolein. 4-hydroxycyclophosphamide, the primary metabolite, is not as biologically active.

Benzene also requires metabolism in order to produce bone marrow toxicity; however, the pattern of bioactivation and detoxification is complex, as both induction as well as inhibition of benzene metabolism protect against myelotoxicity. This contradiction can be best explained by elimination and detoxification. Benzene is volatile and rapidly eliminated via the lungs in the absence of metabolism. Induction of nonspecific biotransforming enzymes results in increased metabolite-conjugate formation and increased elimination. Benzene bone marrow toxicity correlates with target organ concentrations of the secondary metabolites, hydroquinone and catechol, not the primary

---

[2] Eastmond et al., 1987

metabolite phenol. The particular susceptibility of the bone marrow to benzene toxicity may relate in part to the high level of myeloperoxidase activity in that tissue. Phenol, catechol and hydroquinone are excellent substrates for peroxidase-mediated reactions, giving rise to SH-reactive benzoquinone metabolites. Administration of phenol and hydroquinone exacerbates toxicity, suggesting phenol-induced stimulation of hydroquinone metabolism and benzoquinone formation. More recently, the formation of trans-muconaldehyde has also been suggested as the toxicant responsible for benzene toxicity, as it has reactive properties very similar to the quinone metabolites of benzene. Despite marked differences in experimental design or routes of exposure, chronic target organ toxicity is restricted to peroxidase-rich tissues such as bone marrow, Zymbal, Harderian and mammary glands.

Nitroreduction has been implicated in the bioactivation of several nitroaromatic compounds including nitrobenzene and dinitrotoluene. The production of methemoglobinemia by these agents is largely dependent on nitroreduction by bacteria in the gut, a process required for the bioactivation of chloramphenicol. Chloramphenicol causes a dose-dependent myelosuppression; however, the relationship between this reversible phenomenon and the occasional "idiosyncratic" chloramphenicol-induced irreversible aplastic anemia is not clear. Nitroso-chloramphenicol produces an irreversible inhibition of CFU-S in mice in vivo and CFU-C in human bone marrow cells in vitro, indicating this compound is the most likely toxicant.

A tendency for hematotoxic compounds to give rise to very stable metabolites of intermediate reactivity, allowing transport to tissues distant from the site of primary metabolism (i.e. from the liver to the bone marrow),

is a constant finding in hematotoxicity. This observation has most recently been illustrated by the potent murine leukemogen, 1,4-butadiene for which the bone marrow is a major target organ. Although primary metabolism of the compound occurs predominantly in the liver, the major metabolites, monoepoxybutene and diepoxibutane, possess long half lives relative to most biologically active aromatic and aliphatic epoxides.

#1 HD Mechanisms of Toxicity

Hematotoxicity is manifested by altered circulating mature blood cells, both in number and/or interference with their function. A reduction in the number of circulating blood cells results either from excessive destruction (e.g. acquired hemolytic anemia) or suppression of production. These lesions are classified as granulocytopenia, thrombocytopenia, lymphocytopenia or pancytopenia, depending on the cell type predominantly affected.

Anemia is a decrease in the number of circulating RBC, a decrease in their corpuscular volume (MCV), a reduction in hemoglobin content (MCH) or any combination thereof. Anemias can result from increased destruction of erythrocytes, decreased production of erythrocytes, or both, and are usually classified according to the predominant pathology. In making a differential diagnosis of anemia it is important to consider causes of anemia, other than xenobiotic induced, eg. acute blood loss, nutritional deficiencies (eg. iron, vitamin B12 or folate) and congenital abnormalities of the erythrocyte membrane hemoglobin synthesis or metabolism. Certain xenobiotics can act as metabolic antagonists, producing anemias in some species similar to those encountered in nutritional deficiencies or pernicious anemia in man (eg.

aminopterin induced megaloblastic anemia). Finally, anemia is frequently an early secondary finding in leukemias and myelodysplasias.

Decreases in one or more of the leukocyte series or platelets are classified as granulocytopenia, lymphocytopenia or thrombocytopenia, respectively. Because of complex transit kinetics and the small proportion of granulocytes and lymphocytes in the circulation relative to storage compartments, transient fluctuations in the numbers of circulating cells are common. Distinguishing between transient cytopenias resulting from non-specific toxicity and those occurring as a result of specific toxicity to the blood and bone marrow is difficult. Abnormal increases in circulating cells can vary in severity from a transient granulocytosis resulting from demargination to acute leukemia.

Depending on the nature, extent, and chronicity of the injury, bone marrow toxicity may range from a transient suppression of one or more cell type with rapid recovery following cessation of exposure, to myelodysplasia in which blood dyscrasias and aberrant stem cell regulation may persist or progress to aplastic anemia or leukemia.

#2 HD Direct toxicity to circulating cells

#3 HD Hemolytic Anemia

Acquired hemolytic anemia is among the most frequently reported drug-reaction in man, but is usually of minor severity. Decreased lifespan of circulating erythrocytes defines hemolytic anemias, and can range from subtle RBC membrane changes resulting in premature removal by phagocytic cells, to

rupture with a subsequent life threatening hemolytic crisis. It is common to see a minor hemolytic component in bone marrow suppression type anemias.

#4 HD Diagnosis

Laboratory findings in hemolytic anemia vary according to the severity and cause of the disease. Plasma or serum hemoglobin concentration are still the most sensitive indicators in routine use; however, decreased erythrocyte survival time, as measured by non-routine isotopic labelling techniques, may be the only finding. Although increased in unconjugated bilirubin may be seen following increased hemoglobin catabolism, it is neither a very sensitive or specific indicator of hemolytic anemia. If the rate of hemolysis is significant, a decrease in RBC and Hct (or PCV) may be observed. Hemolytic anemias are often accompanied by a regenerative response manifested by hyperplasia of the bone marrow and increased circulating reticulocytes, which may increase the MCV as reticulocytes are larger than mature RBC.

The peripheral blood smear may show morphologic evidence of erythrocyte destruction including erythrocyte fragments (schizocytes), cells exhibiting loss of membrane (spherocytes), RBC agglutination, or Heinz body formation, depending on the pathogenesis of the anemia. Because schizocytes and spherocytes have abnormally low cell volumes, the MCV may not be abnormal as these small cells are balanced by reticulocytes. The coefficient of variation in RBC size may increase dramatically.

Splenomegaly is frequently encountered in chronic hemolytic anemias in rodents because of sequestration of damaged erythrocytes and the presence of

extramedullary hematopoiesis. Clusters of erythroid precursor cells may be found in the red pulp along with hemosiderin-laden macrophages; the entire red pulp may be occupied with erythroid precursors - care must be taken not to confuse the histologic picture with that of lymphoma. Although the presence of basophilic and polychromatophilic erythroblasts can usually be discerned in routine hematoxylin-eosin stained sections, Wright-Giemsa stained sections or imprints are useful for cell identification. Heinz bodies, intracorpuscular precipitates of denatured hemoglobin, indicate oxidative damage to hemoglobin. They constitute the major cause of erythrocyte destruction in hemolytic anemias associated with glucose-6-phosphate dehydrogenase deficiency.

A number of attempts have been made to characterize animal models of hemolytic anemia associated with genetic deficiencies, but many have proved disappointing as experimental surrogates for man. For example, sheep have been characterized with 10% of normal human G-6-PD, but they are insensitive to drugs normally associated with hemolytic anemia in G-6-PD deficient humans.

#4 HD Mechanisms of chemically induced hemolytic anemia

A number of agents are associated with secondary hemolytic anemia in man. Agents are classified as immune mediated, oxidative stress (exposure to potent oxidizing agents, or through increased susceptibility due to congenital glucose-6-phosphate dehydrogenase deficiencies), and non-oxidative. Several excellent detailed reviews are already available on hemoglobinopathies and

hemolytic anemias secondary to drug or chemical exposure in man and will not be repeated here [3]

#3 HD Cytopenias/Cytoses

#4 HD Granulocytopenia.

A number of xenobiotics that alter granulocyte adherence are known to markedly alter the equilibrium between the circulating and marginated compartments. Ethanol, colchicine and epinephrine can rapidly increase the circulating granulocyte pool via demargination, but the resulting granulocytosis does not reflect an increase in host resistance as chemotaxis, phagocytosis and killing are suppressed. Histamine, iron oxide and dextran produce an apparent granulocytopenia by increasing the size of the marginated pool.

#4 HD Thrombocytopenia.

Thrombocytopenia occurs following increased destruction, consumption, aggregation or sequestration of platelets. Large cavernous hemangiomas, massive hepatic necrosis, and hypersplenisn can elicit thrombocytopenia via platelet sequestration and consumption. Hypersplenism induced by methylcellulose injection in rats results in thrombocytopenia which can be corrected following splenectomy.

---

[3] Beutler, 1985; Smith, 1986.

Direct affects on platelet survival are immunologic or non-immunologic, depending on whether antibody complex formation is involved. The precise role of complement-mediated mechanisms of immunologically induced cytopenias is not clear; however, platelets can adsorb complement fixing antibodies by a variety of mechanisms. Xenobiotics can bind to platelets and act as haptens or promote the binding of immune-complexes to platelets by other means. Antibody complex binding can trigger direct complement-mediated platelet lysis or stimulate serotonin release. Alternatively, platelets binding C_3 and Ig are sequestered and phagocytosed. Agents thought to act directly on platelets include the antibiotic, ristocetin, which induces agglutination, and heparin which produces a transient thrombocytopenia, though a poorly understood mechanism.

#2 **HD** Bone marrow suppression

#3 **HD** Cell cycle Kinetics

Bone marrow suppression is the most frequent side effect of cancer chemotherapy. Rapidly proliferating cells demonstrate unique susceptibility to certain cytotoxic drugs compared to their resting counterparts, an effect attributed to their relative position in the cell replicative cycle. The cell cycle can be divided into four phases relative to DNA synthesis: M, mitosis, (physical cell division); G_1, the first gap or period of no measurable DNA synthesis; S, the phase of active DNA synthesis; G_2, the second gap after DNA synthesis is complete, in which the cell contains twice the diploid content of DNA found in G_2. Cells not in active cycle containing the normal diploid content of DNA reside in the G_o phase (Figure 3). G_1 can be further divided

into subcompartments relative to quantitative and qualitative differences in RNA and protein synthesis.

[Please insert Figure 3 & 4 here]

The committed stem cell populations of the bone marrow exhibit a pattern of asynchronous exponential growth, in which cycling cells move through the cell cycle independent of one another, so the probability that a cycling cell will be in any particular phase of the cycle at a given point in time is proportional to the amount of the replication cycle that is spent in that phase. The proportion of asynchronously dividing cells in any given phase can be depicted by a histogram based on the DNA content of cells (Figure 4).

Although many agents are selectively toxic to rapidly dividing cell populations, there can be considerable variation in the response of proliferating precursors, progenitor and stem cell populations to toxicity. Some agents show specificity for stem cell populations which favour self-renewal. Potent alkylating agents, such as busulfan or the nitrosureas, target resting PSC in addition to other proliferating cells, thereby suppressing PSC function and number. Nitrosureas produce delayed and severe bone marrow damage, 1-2 months after administration. Similarly, residual stem cell injury occurs long after cessation of treatment with busulfan.

Stem cell populations in S phase are more resistant to radiation injury than are resting cells, whereas cyclophosphamide preferentially affects stem cells with low self-renewal potential and high proliferative activity. Other alkylating agents, such as actinomycin D are similarly classified as cycle-

specific agents; they selectively target cycling cells in any phase. Bichloro-ethyl-amines, mephalan, chlorambucil and nitrogen mustard, are examples of compounds that are also cycle-specific. These agents typically produce a dose-dependent bone marrow suppression that is often followed by protracted recovery.

Some compounds such as colchicine, the vinca alkaloids, and methotrexate, target cells in a specific phase of the cell cycle, and produce a plateau-type of dose response curve. Colchicine and the vinca alkaloids interfere with microtubule assembly and mitotic spindle formation, which results in an arrest of cycling cells in G_2/M. Susceptibility is confined to cells in late S and early G_1 phase at the time of exposure. Since a proportion of asynchronously dividing cells are in this part of the cycle at any given time, increasing the dose of a phase-specific cytotoxic agent beyond an effective threshold does not increase the number of affected cells (Figure 4).

For cycle-or phase-specific agents, the frequency and duration of exposure can be an important factor in determining toxicity, because altering the timing of exposure relative to cell cycle (eg. varying the interval between doses) can lead to synchronization of the cell cycle, with subsequent alteration of the number of cells susceptible to toxicity at any one time. For example, a single therapeutic dose of cyclophosphamide can result in a transient depression in circulating leukocytes, whereas multiple dosing at two week intervals may result in severe leukopenia, aplastic anemia and death.

Benzene appears to share characteristics in common with both cycle-specific and phase-specific agents. It causes an arrest of cycling cells in G_2/M. This arrest is seen as an initial increase, followed shortly by a decrease in bone marrow cell turnover, suggesting a defect in the maturation of precursor cells. Hydroquinone and its terminal oxidation product, p-benzoquinone, have been implicated as the benzene metabolites responsible for the effects of benzene on proliferating cells. These compounds arrest cycling cells in G_2/M by inhibiting microtubule assembly, thereby interfering with sulfhydryl-dependent GTP binding to the tubulin dimer.

The effects of benzene are not entirely restricted to cells in G, or M phases of the cell cycle, however. At non-cytotoxic concentrations, the quinone metabolites of benzene can inhibit the entry of resting cells into cycle, thus modulating the dose response relationship between responding cells and proliferative stimuli. Direct effects on the cytoskeleton and/or interference have been hypothesized to explain these effects.

Benzene produces a marked lymphocytopenia and suppression of progenitor B-lymphocytes in mice. In addition, hydroquinone reduces the ability of stromal cultures to support granulopoiesis, apparently because of a selective suppression of IL-1 release by macrophages, which results in a reduction in IL-1 dependent IL-4 release by fibroblasts and subsequent pre-B cell suppression.

Nitrosourea-induced bone marrow toxicity has been reproduced in a wide variety of animal models; however, relative differences in species susceptibility to these compounds exist. Mice and cats appear to be

particularly susceptible to the effects of nitrosourea compounds on bone marrow, while rats, guinea pigs and sheep appear to be somewhat resistant. A definitive mechanism of nitrosourea-induced bone marrow toxicity has not been established, but direct alkylation or carbamylation of DNA and RNA have been implicated as potential mechanisms. In structure activity studies between the chloroethylnitrosureas, which produce significant bone marrow toxicity at therapeutic doses, and chlorozotocin, which does not, carbamylation and the regional pattern of alkylation of transcriptionally active chromatin, have been found to correlate with differences in bone marrow toxicity.

#3 HD Megaloblastic Anemia

Erythrocytes are less susceptible than leukocytes to numerical alterations, resulting from interference to hematopoiesis, as they have a long lifespan relative to leukocytes. Therefore, frank anemia is usually a late manifestation of bone marrow suppression, even though erythropoiesis **per se** may be more sensitive to chemical toxicity than granulopoiesis. Bone marrow suppression related anemias rarely occur in the absence of accompanying cytopenia(s) and maturational or "megaloblastic" changes in circulating blood cells. Xenobiotics that interfere with RNA or DNA synthesis, or produce disturbances in cell division frequently produce a megaloblastic anemia. Such agents include folate antagonists (methotrexate), inhibitors of purine or pyrimidine synthesis (6-mercaptopurine and 5-fluorouracil) and pentose sugar analogues such as cytosine arabinoside.

#4 HD Diagnosis

Macrocytic and microcytic erythrocytes can be present in megaloblastic anemias that arise from bone marrow suppression. Distinctive morphologic changes can be seen in precursor cells of erythroid, granulocytic and megakaryocytic lineages in bone marrow; increase in the number of immature progenitor cells is also present. Nuclei may be abnormally large and possess a delicate chromatin pattern, even in cells with relatively mature cytoplasmic features. Precursor cells in all lineages, and circulating erythrocytes, may exhibit increased numbers of micronuclei or Howell-Jolly bodies, a reliable indicator of dyserythropoiesis. These finding are particularly evident in the mouse, as micronuclei-containing cells are not effectively removed by the spleen. The frequency of circulating reticulocytes may be normal or reduced. Circulating hypersegmented granulocytes may be seen, although the number of granulocytes or platelets may not be significantly reduced.

#3 HD **Cytopenias**

Reductions in the number of circulating platelets, granulocytes or lymphocytes are the most common manifestations of bone marrow suppression. However, results may vary, depending on the experimental conditions, the strain or species used, and even the individual animal.

Thrornbocytopenias may result from destruction, decreased production or release of platelets, and are the most frequent blood dyscrasias reported secondary to xenobiotic exposure. A wide variety of drugs have been implicated in immune-mediated thrombocytopenia in man, including **quinine** and

heparin, but animal models are not well defined. Thrombocytopenia, with or without leukopenia, is often encountered following exposure to myelosuppressive agents, although the mechanisms remain largely unknown. Species differences exist as to the outcome of toxicity. Cytosine arabinoside and busulfan, for example, are extremely effective in inducing experimental thrombocytopenia in a wide variety of species. On the other hand, cyclophosphamide administration to BDF1 mice has been reported to produce a marked depression in the number of bone marrow megakaryocytes and **CFJ-M** with only a slight decrease in blood platelets. In contrast, administration of a single low dose of vincristine to BALB/c mice has been reported to result in thrombocytosis without prior thrombocytopenia, whereas high doses of vinblastine, but not bleomycin, produce a transient thrombocytopenia in rats. The dog appears to be sensitive to estrogen-induced thrombocytopenia, presumably mediated through decreased megakaryocytopoiesis, although an immune-mediated mechanism has been suggested for diethylstilbesterol induced thrombocytopenia in this species.

Granulocytopenia may be a useful indicator of myelotoxicity; however, fluctuations in granulocyte numbers frequently occur in experimental animals independent of drug-induced bone marrow suppression. Non-specific stress, or systemic chemical toxicity can transiently alter cell cycle kinetics in proliferating bone marrow populations, producing wide fluctuations in circulating granulocyte numbers. Rodents and rabbits are especially susceptible to stress-induced leukocytopenias. These have been attributed to relative species sensitivity to corticosteroid release, although the precise mechanisms are not understood. For this reason care should be taken in the interpretation of acute or transient fluctuations in leukocyte numbers

observed within a few days of treatment. Dose dependent responses are more important. For example, lymphocytopenia appears to be the most sensitive and reliable indicator of repeated benzene exposure in a variety of species. Surprisingly, granulocytes are relatively resistant to benzene, and granulocytopenia is a late manifestation of benzene toxicity.

#3 HD Aplastic anemia

Aplastic or non-regenerative anemia is a syndrome associated with bone marrow failure, characterized by anemia, pancytopenia, and varying degrees of bone marrow hypocellularity. It is distinguished from other diseases producing pancytopenia, such as leukemia, infiltrating malignancies, or myeloproliferative disease, by histologic examination of the bone marrow. Histologically, hematopoietic precursor cells may be almost totally replaced by fat, leaving a few mature hematopoietic cells interspersed throughout (Figure 5).

[Please insert Figure 5 here]

Aplastic anemia is classified as idiopathic or secondary depending on whether its onset can be attributed to known causes, eg. radiation, drug or chemical exposure. The syndrome carries a grave prognosis, as in the absence of successful bone marrow transplantation, approximately 40% of all human cases die within 6 months of diagnosis. Aplastic anemias arising secondarily to chemotherapy or xenobiotic exposure carry an even more dismal prognosis.

Aplastic anemia is a disorder of stem cell regulation. Differing theories on the pathogenesis of the disease emphasize the respective roles of hematopoietic stem cells and stromal cells in its evolution. Aplastic anemia has been suggested as a disorder of PSC which, either through exhaustion of numbers or a defect in differentiation, are unable to recapitulate blood cells. There is also evidence that stromal cell defects may play an important role in chronic bone marrow failure, but the success of HLA-matched allogenic marrow transplants in man, and the inability of growth factors to ameliorate the disease, suggests that microenvironmental defects alone cannot completely explain aplastic anemia. In many cases of aplastic anemia, the hypoplastic marrow is devoid of hematopoietic precursor cells; however, residual islets of erythroid or granulocytic precursor cells may persist. In some of these cases there is evidence to support a clonal origin for aplastic anemia.

Animal models of aplastic anemia are relatively few, and have been largely restricted to those induced by viruses, busulfan, irradiation or benzene. A problem frequently encountered is animals die due to anemia, hemorrhage or infection, before fulfilling the stringent criteria for a diagnosis of aplastic anemia. A rapidly developing neutropenia, thrombocytopenia and anemia accompanied by a decrease in bone marrow cellularity of CBA mice has been described following infection with Rauscher leukemia virus. Morphological examination of bone marrow from these animals revealed a maturation arrest in the granulocyte series. It was concluded that the underlying mechanism involved a disorder in differentiation, probably at the level of a common precursor cell.

Busulfan-induced aplastic anemia is the best studied model of chemically-induced bone marrow failure. In Swiss mice given an initial treatment of four doses, busulfan produced a transient bone marrow hypoplasia followed by a normal blood picture; by 240 days 80% of the mice died with pancytopenia. In some instances, no differences in bone marrow cellularity or peripheral blood counts were noted during the latent phase, although lymphocytopenia was a common finding. Subsequent studies revealed that CFU-S and granulocyte progenitor cells were decreased fol1owing busulfan treatment, and were further suppressed following frank bone marrow failure. In reconstitution studies, bone marrow cells from busulfan treated mice grew poorly when inoculated into irradiated mice, providing additional support for a stem cell defect. In addition, evidence for residual stromal damage was evidenced by persistence of a maturational defect following "correction" of the aplasia in irradiated busulfan-treated mice.

For over fifty years the bone marrow has been recognized as being particularly susceptible to radiation injury and a cause of aplastic anemia in mice. The dog has been used as a model for radiation-induced aplastic anemia figuring prominently in models used for bone marrow transplantation. Radiation produces both transient and prolonged bone marrow suppression, depending on the dose, dose rate, and exposure conditions. Local irradiation of rats results in a transient hypoplasia and recovery following 2000 rds, whereas twice the dose results in a transient hypoplasia, with a latent period followed by a prolonged hypoplasia. Aplastic anemia resulting from either busulfan or irradiation is characterized by a marked reduction in CFU-S, BFU-E, CFU-E and CFU-GM in mice.

Despite the unquestioned potential of benzene to produce bone marrow damage in man, animal models of benzene-induced aplastic anemia remain difficult to produce. Experimental evidence has been contradictory, in some instances; however, examination of these findings in conjunction with those previously described for the effects of benzene metabolites on macrophage function indicate that benzene exposure results in prolonged stem cell abnormalities, both abnormal differentiation and damage to the stromal cell populations.

#3 HD Myelofibrosis

Following injury the bone marrow may undergo necrosis followed by replacement by fibrosis. This reaction may become progressive, resulting in virtually total displacement of hematopoietic precursor cells with fibrous tissue. Myelofibrosis in man is now classified as a myeloproliferative disease according to the International Classification of Diseases. Based on studies of saponin-induced myelofibrosis in rabbits, it has been hypothesized that the pathogenesis of the disease predominantly involves interference with the blood supply to the bone marrow. Hematologic findings vary considerably, depending on the species and the severity of the lesion; however, the spleen is usually enlarged, with myeloid metaplasia a predominant feature.

#2 HD Clonal Hemopathies

Recognition of the existence and nature of the clonal hemopathies resulted from the integration of two independent lines of experience in clinical and experimental hematology. Firstly, it has been recognized that

leukemia in man is frequently preceded by progressive hematologic abnormalities (hematopoietic dysplasias), which represent a spectrum or continuum of abnormalities rather than separate or unrelated entities. Secondly, considerable evidence suggests these dysplasias, collectively termed myelodyplastic syndrome **(MDS)**, and leukemias are predominantly clonal hemopathies, sharing a common stem cell target.

#3 HD Myelodysplastic syndrome (MDS)

It has been recognized for nearly thirty years that cases of leukemia, primarily acute myelogenous leukemia, are often preceded by a "preleukemic" or prodromal state of chronic bone marrow insufficiency. This phenomenon occurs with alarming frequency in leukemias arising secondarily to previous cancer therapy. A number of studies have also shown that MDS often precedes lymphoproliferative disorders.

MDS can be manifested as either bone marrow insufficiency or a proliferative disorder. Insufficiency is associated with varying degrees of anemia, usually accompanied by macrocytosis, and leukopenia or thrombocytopenia. Histologically, the bone marrow is usually hypocellular or an excessive proliferation of one or more cell lineages can be observed. The rate of conversion of MDS to frank leukemia in man appears to be particularly high in cases arising secondary to chemotherapy or benzene exposure. Studies utilizing cytogenetic or biochemical markers to identify the clonal origins of abnormal cell populations have revealed that clonal abnormalities may be present for years prior to the clinical onset of leukemia. MDS, itself carries a grave prognosis with mortalities reported of 30 - **84%.**

Experimentally induced MDS has been studied in dogs, rats and mice. Dogs receiving a leukemogenic dose of radiation developed acute nonlymphocytic leukemia preceded by changes identical to MDS in man. During the recovery phase animals exhibited macrocytic anemia, thrombocytopenia and neutropenia. Bone marrow exhibited hypercellularity and severe hematopoietic dysplastic changes. Persistent dysplastic myeloproliferative lesions have been reported in rats following treatment with dimethylbenz(a)anthracene, and in mice following exposure to benzene. In both species signs of hematopoietic dysplasia were most obvious in the spleen, consistent with its role as an extramedullary hematopoietic organ in rodents. Hyperplastic and dysplastic changes in the erythroid series were prominent in rats, whereas, myeloid dysplasia predominated in mice. A macrocytic anemia was observed in most mice, and hyperplastic changes in the spleen were prominent despite the presence of a persisting anemia. Abnormalities were noted in the number and morphology of granulocytic, megakaryocytic and erythroid precursor cells, including nuclear deformities and atypical mitotic figures.

#3 HD Leukemia

Leukemias are among the most widely recognized and feared malignancies in man. Derived from bone marrow, they are usually disseminated via the blood at some time during their development. In general, leukemias can arise as abnormal cell populations from any cell lineage, although there is some predisposition to one or more cell types, depending on the method of induction and the species or strain studied. In the United States and Europe, leukemias

in man associated with chemical or drug exposure are predominantly myeloid, although secondary lymphoid leukemias and lymphomas are by no means rare.

With the exception of certain strains, eg. CBA/Ca; mice exhibit a predisposition toward lymphoid neoplasms. Spontaneous leukemias or viral-induced have been described in virtually every mammalian species examined. A comparative discussion of spontaneous leukemias across species is beyond the scope of this chapter. Because of the singular prominence of the rodent in chemical leukemogenesis studies, the focus here will be on the rat and mouse.

The abnormal cell population tends to be arrested at a single stage of differentiation in most leukemias and lymphomas. Although there are exceptions, leukemias consisting predominantly of immature or blast cells tend to proliferate rapidly and be aggressive, whereas those in which a more differentiated phenotype prevails, tend to take a more protracted course.

A common feature of hematologic malignancies in both experimental animals and man is their clonal nature, i.e. they are derived from a single cell. Lymphoid and myeloid stem cells are consistently involved as the targeted cell compartment in leukemias of lymphoid and myeloid origin. As a result, the biologic significance concerning differences among acute lymphoid and myeloid leukemias amongst species, may prove to be less important than were historically assumed. On the other hand, chronic myelogenous leukemia in man, originates in an earlier cell, perhaps the PSC. It is apparent, therefore, that elucidation of the mechanism(s) of leukemogenesis is inextricably linked to an understanding of stem cell regulation. Leukemogenesis is a multi-factorial process which cannot be modeled or

simulated as a function of a uni- or a bimolecular event. Therefore, it is not surprising that no initiation-promotion paradigms have been established for chemical leukemogenesis in experimental animals.

#4 HD Diagnosis

Leukemia and/or lymphoma has been defined as "[increased] number of specific hematopoietic cells in the absence of a demonstrable, appropriate stimulus. Cellular excess may be localized to a single lymph node, ... or may be quite widespread and involve bone marrow, blood and many lymph nodes as well as other organs..."[4]. This definition belies the difficulty which often obscures the diagnosis of leukemia, especially in rodents which constitute the most frequently used models in experimental carcinogenesis. The importance of distinguishing between leukemias and lymphomas and other "non-malignant" dysplasias of the hematopoietic system has been exaggerated in government regulatory policy, where an almost mystical significance appears to be attached to the diagnosis of leukemia. It should be noted that the biological significance of a differential diagnosis of myeloid leukemia versus myelodysplasia to the host is virtually nil.

The distinction between lymphoma, a malignancy involving primarily the thymus, lymph nodes or splenic white pulp, and leukemia, a malignancy involving primarily the bone marrow, spleen or blood is often difficult or unwarranted. Mouse lymphoid neoplasms form space occupying lesions, yet spread in a leukemoid manner, rendering the distinction difficult.

---

[4] **Witnrobe, 1974**

Granulocytic neoplasms usually present as blood borne neoplasms in most species, whereas they exhibit a significant tendency to spread as solid tumors in rodents. While these distinctions may be of little biological significance, they can be important considerations in the interpretation of tumor incidence data derived from animal studies in which different classification schemes are used.

#5 HD Rat

Spontaneous leukemias are rare in most strains of laboratory rat. This species is also relatively resistant to chemically-induced leukemogenesis. Both lymphoid leukemia and chloroleukemia have been reported in Wistar rats following the administration of methylcholanthrene. Although spontaneous granulocytic leukemias have been reported in Sprague Dawley rats, they are rare. Large granular cell leukemia (LGL) arises in 10 - 50% of Fischer **344** rats over **18** months of age limits their usefulness in lifetime bioassays. First recognized and described as a specific entity in Wistar/Furth and Fischer rats, this neoplasm constitutes a major cause of death in **F344** rats 2 years of age or older. Compounds, such as allyl isovalerate and ethylene oxide, have been demonstrated to increase the incidence of LGL leukemia in **F344** rats; however, controversy **exists** as to whether this increase represents a leukemogenic response or an altered onset of a spontaneously occurring tumor. Morphologically, LGL cells resemble large granular lymphocytes and have been identified as natural killer cell of the rat. The neoplasm arises in the spleen and spreads to the bone marrow late in the course of the disease. LGL is accompanied by splenomegaly and an autoimmune hemolytic

anemia. Erythrophagocytosis is a common feature in LGL infiltrates in the spleen.

#5 HD Mouse

Many classification schemes of murine lymphoid neoplasms have been described. One of the most widely adopted classifications is that proposed by Dunn[5] which separates lymphoid neoplasms into lymphocytic, reticulum cell types A and 8, and plasma cell lymphomas, based solely on morphology. Pattengale and Taylor[6] introduced an immuno-morphologic classification of murine lymphomas. Both of these schemes provide detailed criteria for the diagnosis of histocytic and 8-cell lymphomas, which constitute the vast majority of spontaneous lymphomas encountered in most mouse strains. In contrast, thymic (T cell) lymphoma/leukemia occurs rarely in most murine strains. The AKR and **C58** mouse strains are principal exceptions in which thymic lymphoma appears spontaneously within the first year of life and is closely associated with endogenous ecotropic retrovirus. Thymic lymphoma is the principal neoplasm associated with radiation or xenobiotic exposure in a variety of strains, including C57BL/6,, RF, and C3H mice. The respective roles of chemical exposure and retroviruses in these murine models remains the subject of debate.

Regardless of the classification scheme employed, care must be taken to use comparable diagnostic criteria when comparing the results of different

---

[5] Dunn, 1954

[6] Pattengale and Taylor, 1981; 1983

studies. The B6C3F1 hybrid (C57BL/6 x C3H) mouse has become the standard murine model for chemical toxicity and carcinogenicity studies, because of the low incidence of spontaneous lymphomas encountered in this hybrid, relative to other mouse strains. Nevertheless, over a two year lifespan, the incidence of spontaneous lymphoid neoplasms is 8.3% in males and 16.8% in females. These tumors arise late in life, most frequently in lymph nodes, spleen or liver, and are presumably of B-cell origin. The incidence of spontaneous thymic lymphoma in these mice is negligible. The incidence of lymphoma in B6C3F1 mice chronically exposed to 1,3-butadiene is high, approaching 60% in males by the end of one year. In contrast to spontaneous lymphomas, these tumors are mediastinal in origin and uniformly express T-cell surface markers. Although butadiene would be classified as a murine leukemogen, it is appropriate to discuss thymic lymphomas in the context of a negligible historical incidence. To date, few chronic studies have employed sufficient diagnostic criteria to make such distinctions.

Differentiating hyperplastic versus neoplastic lymphoid lesions is difficult, particularly in mice, as tissue availability is limited. Conservative criteria should be used, since normal lymphoid populations possess some characteristics suggestive of malignant cells in solid tissues; normal lymphoid populations retain the capacity to divide, have the ability to invade lymphoid and non-lymphoid tissues, and can kill targeted cells. As a result, reactive hyperplastic responses can be extremely difficult to distinguish from truly malignant neoplasms by histopathologic analysis alone. When in doubt, successful transplantation of tumor cells into syngeneic or nude hosts remains the sine qua non for demonstrating malignancy.

The diagnosis of murine myeloid leukemia is also difficult, as myeloproliferative disease is characterized by features often commonly encountered in both neoplastic and acute inflammatory processes. Under conditions of acute inflammation, or hematopoietic stress, the spleen is the first site of extramedullary hematopoiesis. It is also frequently the primary site for the development of myelogenous leukemia. To confuse matters further, both processes can be accompanied by extensive myeloid hyperplasia in the red pulp of the spleen. The early infiltration into surrounding tissues by leukemic cells is similar to that encountered in extramedullary granulopoiesis in response to acute inflammation.

Various authors have differed widely in their criteria for diagnosis of leukemia in mice. Dunn[7] suggested using the lack of a local inflammatory reaction and the presence of collections of relatively immature granulocytic or blast cells as diagnostic criteria, particularly in areas where extramedullary hematopoiesis is seldom found. Unfortunately, neoplastic proliferation in the mouse is often characterized by a variable range of maturation in the myeloid series, so Barnes and Sisman[8] concluded that a predominance of blast cells and ring forms are very reliable criteria for distinguishing neoplastic from compensatory hyperplasia.

Although total replacement of lymphoid follicles is a distinguishing feature in cases of myeloid leukemia, some follicular remnants are almost always spared. Because murine myeloid tumors exhibit a tendency to spread

---

[7] Dunn, 1954

[8] Barnes and Sisman, 1939

either by direct extension or emboli, and acute inflammation is accompanied by a marked neutrophilia in mice, leukocytosis cannot be considered a reliable diagnostic sign. In cases where the disease is largely limited to the spleen, the predominance of immature cells in conjunction with intravascular extension or spread may be an important morphologic differentiating factor. In the final analysis, the distinction between a malignant and non-malignant myeloproliferative lesion is definitely not black and white.

#1 HD Methodology for the Evaluation of Bone Marrow Toxicity

Increasingly sophisticated automated methods for the identification of individual cells, long term bone marrow cell culture, and technologies for cell and molecular cloning have led to an explosion the number of methods available for the study of toxicity to the hematopoietic system. The purpose of this section is to provide a general discussion of the advantages and potential limitations of various approaches used to evaluating toxicity to the blood and bone marrow. Detailed discussions of individual methods and applications are readily available elsewhere in the literature.

#2 HD Evaluation of Toxicity

#3 HD Clinical Laboratory Hematology

Analysis of the blood remains the most widely employed method for evaluating hematotoxicity of chemicals and physical agents. Most procedures commonly employed in the human clinical laboratory have been adapted for use in experimental animals. The complete blood count (CBC) typically includes: the leukocyte count (WBC), erythrocyte or red cell count (RBC), hemoglobin

(HGB), hematocrit or packed cell volume (HCT or PCV), platelet count (PLT), and erythrocyte indices, ie. mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC), morphologic examination of the peripheral blood smear, and differential leukocyte and reticulocyte counts. With more advanced techno1ogy, parameters such as the red cell distribution width (RDW), are now available. Procedures used for the diagnosis of human clotting disorders have also been adapted for use in experimental animals.

Descriptions of both manual and automated methods, and standardization of these procedures, are available in any hematology reference. Most commercially available instrumentation for automated analysis of blood can be adapted for experiments which often have small samples. The limitations of small sample size cannot be overemphasized when using rodents, especially mice!

Sampling site and collection method influence hematology values; therefore, site is another important consideration in the design, interpretation and comparison of hematologic studies in rodents. Sampling sites can be a major source of variation between experiments. Sources commonly used include: tail, orbital plexus, abdominal aorta, abdominal vena cava, and heart. Tail blood WBD are approximately twice that of abdominal aorta and significantly higher than those from the orbital plexus. Although the orbital plexus represents a frequent site for serial sampling of blood from rodents, it should be recognized that such samples are primarily capillary in origin and differ from other sites with respect to WBC, RBC, HGB,

HCT and differential counts. Furthermore, samples taken from the orbital plexus vary depending on the operator's technique.

In addition site having an effect on hematologic parameters, it also important in determining what volume can be collected. The order of collection can account for differences; hematology samples should be drawn first. In general, hamatological parameters measured from right ventricular blood tend to be the least variable and most consistent.

#3 HD Bone **Marrow** Cellularity

Bone marrow cellularity, being relatively independent of the transient fluctuations in blood leukocytes and platelets, is a useful adjunct to the complete blood count. Quantitative methodology is considerably more sensitive than histopathologic analysis. It is possible to detect changes of **25%** of a certain cell type using quantitative methodology, whereas a change of 80% or more is required for reliable detection by histopathology. A variety of procedures quantify bone marrow cellularity including: hemocytometers, automated cell counters, and the use of ^{59}Fe uptake. Cellularity is usually expressed in terms of the total number of nucleated cells per milligram, in rats or larger animals; the total number of cells per tibia or femur can be determined in mice.

#3 HD **Flow Cytometry**

Techniques such as flow cytometry, in conjunction with modern cell and molecular biology, have revolutionized experimental and clinical hematology.

Flow cytometry allows high speed multiparameter analysis and purification of individual cells in suspension. Fluorescence detection methods have been successfully applied to DNA and RNA/DNA for cell cycle analysis, cytotoxicity, phagocytosis, and cell identification (using surface marker analysis). Cell sorting based on surface marker analysis has recently been coupled with mechanical cell purification technology for the successful purification of murine PSC.

#3 HD Ferrokinetics

Ferrokinetic analysis? measuring ^{59}Fe utilization, has occasionally been used to monitor chemical toxicity to the bone marrow in rodents. It is a crude indicator of bone marrow function. Nevertheless, ferrokinetic data in rodents must be interpreted with caution for a variety of reasons: Firstly, incorporation of iron into erythroid precursors is not uniform. Uptake is greatest in immature erythroid cells and diminishes with progressive differentiation. Secondly, the use of ferrokinetic data to estimate the differential effects of chemical toxicity on various erythroid precursor compartments assumes a constant hematopoietic tissue mass. This is a problem which cannot be control1ed for in rodents because of extramedullary hematopoiesis. Thirdly, under non-steady state conditions the number of divisions necessary to produce reticulocytes may vary. Finally, the premature loss of erythrocyte precursors, ie. "ineffective erythropoiesis", may vary with toxicity or proliferative response to blood loss.

#2 HD Analysis of stem cell populations.

#3 HD **CFU-S**

The traditional in vivo mouse spleen-colony forming assay (CFU-S) remains the standard method to quantitatively assess PSC in mice. The assay assumes that multipotential stem cells will give rise to colonies of hematopoietic cells in the spleen of lethally irradiated mice, following inoculation with a limiting number of bone marrow cells. Morphologic evaluation of these colonies reveals erythrocytic, granulocytic, megakaryocytic, or mixed cell type colonies. Mixed cell colonies are indicative of a multipotential precursor cell.

The CFU-S assay is essential for the initial evaluation of stem cell alterations. Nevertheless, the assay possesses certain limitations that must be considered when applying it to the evaluation of chemical toxicity. Firstly, timing of the assay procedure is important. Colonies are apparent in the spleen seven days after reconstitution; however, only colonies forming after 10 - 12 days correlate well with PSC activity. Because survival of lethally irradiated mice beyond 8 days is difficult in the absence of effective barrier facilities, some investigators have chosen to evaluate the effects of chemical pretreatment on CFU-S 8 days after inoculation. Unfortunately, colonies appearing at 8 days post-inoculation represent a disproportionately high percentage of committed erythroid progenitor cells, which more than likely may disappear with time; these do not measure PSC. Secondly, it is now recognized that the PSC compartment itself is heterogeneous, ranging from cells with a high potential for self-replication and less propensity to differentiate (CFU-S-1), to those with relatively

limited ability to replicate and a high tendency to differentiate (CFU-S-II). These represent two major subsets of the PSC compartment that are presumably affected by exposure to myelotoxic agents and that are not readily distinguished using the in vivo CFU-S assay. Thirdly, the CFU-S assay has limited sensitivity, with approximately 5% of injected stem cells colonizing the spleen. Fourthly, the maximum number of colonies that can be reliably distinguished in the mouse spleen before confluence of colonies obscures results is on the order of 12-15. Lack of distinction, coupled with the high mortality in lethally irradiated mice, results in the requirement for large numbers of recipient animals and/or the use of pooled donor cells, which limits experimental design. Finally, the seeding efficiency of active PSC is less than half that of resting PSC, rendering interpretation of CFU-S data difficult in the face of altered stem cell activation resulting from chemical exposure. Thus, the representative subset of PSC measured by **CFU-S** during a compensatory hematopoietic response to bone marrow injury is probably altered over and above a treatment-related effect on the absolute number of PSC. These characteristics of the assay can serve to limit its usefulness or, alternatively restrict the interpretation that can be placed on CFU-S data when used to evaluate mechanisms of stem cell injury.

#3 HD **CFU-Mix**

The in vitro multi-CFC assay is an alternative method which offers some advantages including: increased sensitivity, elimination of interpretive problems related to changes in stem cell splenic colonization efficiency, and elimination of the requirement for using large numbers of lethally irradiated mice. Unfortunately, the potential for self renewal in multi-CFC populations

appears to be extremely low. It is likely that the major cell type enumerated in the CFC mix assay corresponds to CFU-S-II, but it may be feasible to directly d scriminate between CFU-S-I and II in the in vitro assay. An advantage of in vitro bone marrow culture systems is the potential for direct comparison of animal cells and/or with human stem cell populations - a capability obviously not afforded with the use of the mouse CFU-S assay.

#3 HD Progenitor cell assays

Clonigenic assays have been developed to measure virtually every known progenitor cell population, and provide a means to measure the effects of agents on progenitor cell numbers following exposure in vivo. Alternatively, they can be used to evaluate the effects of in vitro exposure to myelotoxic agents on progenitor cell differentiation and proliferation. The use and measurement of defined growth factors makes evaluation of the roles of growth factor elaboration and target cell response in myelotoxicity a possibility.

#3 HD Long Term Bone Marrow Cultures

Techniques and conditions have also been defined that enable the long term culture of pluripotential and progenitor bone marrow cells. Pioneered by Michael Dexter and colleagues in the late 1970's[9], these methods involve the co-culture of hematopoietic and adherent bone marrow stromal cells, creating conditions at least partially analogous to the in vivo microenvironment. Studies using this technique have revealed that the adherent layer is the

---

'Dexter et al., 1982

major site of stem cell origin, which subsequently releases progenitor and differentiating lineage-committed precursor cells . Although the relationships between adherent stromal cells, stem, progenitor cells and their defined growth requirements are necessarily complex in these co-culture systems, they offer considerable promise for the study of the mechanisms of drug and chemical action on regulation of hematopoiesis.

#1 HD SUGGESTED READING

#2 HD Gross and Microscopic Anatomy

Lichtman,M.A. (1981). The ultrastructure of the hemopoietic environment of the marrow. A review. Exp. Hematol. 9, 391-410.

Weiss, L. (1968). Structure of the lymphatic apparatus. Cancer Chemother. Reports. 52, 21-28.

Weiss, L. (1980). The haemopoietic microenvironment of bone marrow: An ultrastructural study of the interactions of blood cells, stroma and blood vessels. In "Blood Cells and Vessel Walls: Functional Interactions" Ciba Foundation Symposium 71, pp. 3-20. Excerpta Medica, Amsterdam.

#2 HD Hematopoeisis Stem and Progenitor Cells

Abramson, S., Miller, R.G., and Phillips, R.A. (1977). The identification in adult bone marrow of pluripotent and restricted stem cells of the myeloid and lymphoid systems. J.Exp.Med. 145, 1567-1579.

Axelrad, A.A., McLeod, D.L., Shreeve, M.M., and Heath, D.S. (1974). Properties of cells that produce erythrocytic colonies in vitro. In "Hemopoiesis in Culture" (W. Robinson, ed.), p. 226. US Gvt. printing office, Washington, D.C.

Barnes, D.W.H., Ford, C.E., Gray, S.M., and Loutit, J.F. (1959). Spontaneous and induced changes in cell populations in heavily irradiated mice. Geneva Conference Paper. 2, 1-10.

Becker, A.J., McCulloch, E.A., and Till, J.E. (1963). Cytological demonstration of the clonal nature of spleen colonies derived from transplanted mouse marrow cells. Nature. 197, 452-454.

Calvo, W., Fliedner, T.M., Herbst, E., Hugl, E., and Bruch, C. (1976). Regeneration of blood forming organs after autologous leukocyte transfusion in lethally irradiated dogs. II. Distribution and cellularity of the marrow in irradiated and transfused animals. Blood. 47, 593-601.

Downey, H. (1938). "Handbook of Hematology". P.B. Hoeber Inc. New York.

Fauser, A.A., and Messner, H.A. (1979). Identification of megakaryocytes, macrophages and neosinophils in colonies of human bone marrow containing neutrophilic granulocytes and erythroblasts. Blood. 53, 1023-1030.

Fialkow, P.J., Denman, A.M., Jacobson, R.J., and Lowenthal, M.N. (1978a). Chronic myelocytic leukemia: origin of some lymphocytes from leukemic stem cells. J. Clin. Inv. 62, 815-849.

Hara, H., and Ogawa, M. **(1978).** Murine hemopoietic colonies in culture containing normoblasts, macrophages, and megakaryocytes. Am. J. Hematology 4, **23-34.**

Jacobson, L.O., Marks, E., and Gsaton, E. **(1954).** Observations on the effect of spleen shielding and the injection of cell suspensions on survival following irradiation. Radiobiology Symposium. **122-133.**

Johnson, G.R., and Metcalf, D. **(1977).** Pure and mixed erythroid colony formation in vitro stimulated by spleen conditioned medium with no detectable erythroprotein. Proc. Nat. Acad. Sci. 74, **3879-3882.**

Johnson, G.R., and Nicola, **N.A. (1984).** Characterization of two populations of CFU-S fractionated from mouse fetal liver by fluorescence-activated cell sorting. J. Cell Physiol. 118, **45-52.**

Lemischka, **I.R.,** Rauler, D.H., and Mulligan, R.C. (1986). Developmental potential and dynamic behavior of hematopoietic stem cells. Cell. **45, 917-927.**

Magli, M.C., Iscove, N.N., and Odartchenko, N. **(1982).** The transient nature of early haemopoietic spleen colonies. Nature 295, **527-529.**

Maximow, A.A. **(1924).** Relation of blood cells to connective tissue and endothelium. Physiol. Rev. 4, **533-563.**

Messner, H.A., and Fauser, A.A. **(1980).** Culture studies of human plruipotent hemopoietic progenitors. Blut. 41, **327-333.**

Messner, H.A., Jamal, N., and Izaguirre, C. **(1982).** The growth of large megakaryocyte colonies from human bone marrow. J. Cell Physiol., Suppl. 1, **45-51.**

Metcalf, D., Johnson, G.R., and Mandel, T.E. **(1979).** Colony formation in agar by multipotential hemopoietic cells. J. Cell Physiology, 98(2), **401-420.**

Metcalf, **D.,** Johnson, **G.R.,** and Nicola, N.A. **(1982).** Separation and commitment of hemopoietic stem and progenitor cells, In "Haemopoietic Stem Cells" (Killman, **SV-AA.,** Cronkite, E.P., Muller-Berat, C.N. eds.), **pp. 29-38.** Munksgaard, Copenhagen.

Molineux, G., Schofield, R., and Testa, N.G. **(1986).** Development of spleen CFU-S colonies from Day **8** to Day **11**: relationship to self-renewal capacity. Exp. Hematol. 14, **710.**

Nakahata, **T.,** and Ogawa, **M. (1982).** Identification in culture of a class of hemopoietic colony-forming units with extensive capability to self-renew and generate multipotential hemopoietic colonies. Proc. Nat. Acad. Sci. 79, **3843-3847.**

Patt, H.M., and Qaustler, H. **(1963).** Radiation effects on cell renewal and nrelated systems. Physiol. Rev. 43, 357-396.

Simminovitch, L., Till, J.E., and McCulloch, E.A. **(1963).** Decline in colony-forming ability of marrow cells subjected to serial transplantation into irradiated mice. J. Cell and Como. Physiol. **64, 23-32.**

Snodgrass, R., and Keller, G. **(1987).** Clonal fluctuation within the haematopoietic system of mice reconstituted with retrovirus-infected stem cells. The EMBO J. 6(13), **3955-3960.**

Spangrude, G.J., Heimfeld, S., and Weissman, I.L. **(1988).** Purification and characterization of mouse hematopoietic stem cells. Science 241, **58-62.**

Till,J.E., and McCulloch,E.A. **(1961).** A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. Radiation Res. 14, **213-222.**

van Bekkum,D.W. **(1977).** The appearance of the multipotential hemopoietic stem cell, In "Experimental Hematology Today" (S.J. Baum and G.D. Ledney, eds.), **p. 3.** Springer Verlag, New **York.**

Visser,J.W.M., Bauman,J.G.J., Mulder,A.H., Eliason,J.F. and deLeeuw,A.M. **(1984).** Isolation of murine pluripotent hemopoietic stem cells. J.Exp.Med. 59, **1576-1590.**

Weed,R.I. **(1970).** The importance **of** erythrocyte deformability. Am.J.Med. 49, **147-150.**

Wilson,F.D. **(1985).** Clonogenic Stem and Progenitor Cell Assays for the evaluation of chemically induced myelotoxicity, In "Toxicology of **the** Blood and Bone Marrow" (R.D. Irons, ed.), pp. **65-100.** Raven Press, New York.

Wu,A.M., Till,J.E., Siminovitch,L., and McCulloch,E.A. **(1967).** A cytological study of the capacitiy for differentiation of normal hemopoietic colony-forming cells. J. Cell. Physiol. 69, **177-184.**

#2 HD Regulation **of** Hematopoiesis.

Bagby,G.C.Jr., McCall,E., and Layman,D.L. **(1983).** Regulation of colony stimulating activity production: Interactions of fibroblasts, mononuclear phagocytes and lactoferrin. J.Clin.Invest. 71, **340-344.**

Bonsdorff,E., and Jalavisto,E. **(1948).** A humoral mechanism in anoxic erythrocytosis. Acta Physiol.Scand. 16, **150-170.**

Broxmeyer,H.E. **(1986).** Biomolecule-cell interactions and the regulation of myelopoiesis. Int.J.Cell Cloning 4, **378-405.**

Broxmeyer,H.E., Williams,D.E., Hangoc,G., Cooper,S., Gillis,S., Shadduck,R.K., and Bicknell,D.C. **(1987).** Synergistic myelopoietic actions in vivo after administration to mice of combinations of purified natural murine colony-stimulating factor 1, recombinant murine interleukin 3, and recombinant murine granulocyte/macrophage colony stimulating factor. Proc.Nat.Acad.Sci. 84, **3871-3874.**

Burgess,A.W., Metcalf,D., Russel,S.H.M., and Nicola,N.A. **(1980).** Granulocyte/macrophage-, megakaryocyte-, eosinophil- and erythroid colony-stimulating factors produced by mouse spleens. Biochem J 185, **301-314.**

Carnot,P., and Deflandre,C. **(1906).** Sur l'activite hemopoietique du serum, C.R. Acad. Sci. (Paris) 143, **384.**

Cronkite,E.P., Harigaya,K., Garnett,H., Miller,M.E., Honikel,L., and Shadduck,R.K. **(1982a).** production of colony-stimulating factor by a murine bone marrow cell line derived from the Dexter adherent layer and other properties of this cell. In "Experimental Hematology Today" pp. **11-18,** S. Karger, New York.

Dexter,T.M., Allen,T.D., and Lajtha,L.G. **(1977).** Conditions controlling the proliferation of haemopoietic stem cells in vitro. J. Cell Physiol. 91, **335-344.**

Dexter,T.M. **(1982).** STromal cell associated hemopoiesis. J. Cell Physiol. (Suppl). 1, **87-94.**

Emerson,S.G., and Gale,R.P., **(1987).** The regulation of hematopoiesis following bone marrow transplantation. Intl. J. Cell Cloning 5, **432-449.**

Harrison,D.E. **(1979).** Use of genetic anemias in mice as tools for haematological research. Clinics in Haematology, 8, **239-247.**

Ihle,J.N., Keller,J., Henderson,L., Klein,F., and Palaszynski,E. (1982a). Procedures for the purification of interleukin **3** to homogeneity. J. Immunol. 129, **2431-2436.**

Ihle,J.N., Keller,J., Greenberger,J.S., Henderson,L., Yetter,R.A., and Morse,H.C. **(1982b).** Phenotypic characteristics of cell lines requiring interleukin **3** for growth. J. Immunol. **129, #4, 1377-1383.**

Jedrzejczak,W.W., Sharkis,S., Ahmed,A., and Sell,K.W. **(1977).** Theta-sensitive cell and erythropoiesis: Identification of a defect in W/W^{v} anemic mice. Science 196, **313-315.**

Kay,H.E.M. **(1965).** How Many Cell-Generations? The Lancet II, **418-419.**

Knospe,W.H., Blom,J., and Crosby,W.H. **(1966).** Regeneration of locally irradiated bone marrow. I. Dose dependent long-term changes in the rat, with particular emphasis upon vascular and stromal reaction. Blood **28,** **398-415.**

Monette,F.C. and DeMello,J.B. **(1979).** The relationship between stem cell seeding efficiency and position in cell cycle. Cell Tissue Kinet. 12(2), **161-175.**

Nathan,D.G., Chess,L., Hillman,D.G., Clark,B., Breard,J., Merler,E., and Housman,D.E. **(1978).** Human erythroidburst-forming unit: T cell requirement for proliferation in vitro. J.Exp.Med. 147, **324-339.**

Reynolds,C.W., Timonen,T., and Herberman,R.B. **(1981).** Natural killer **(NK)** cell activity in the rat. I. Isolation and characterization of the effectorcells. J.Immunol. 127, **282-287.**

Ruscetti,F., Cypess,R., Chervenick,P., **(1975).** Specific release of neutrophil and eosinophil stimulating factors from sensitized lymphocytes. Clin.Res. 23, 406A.

Shadduck,R.K., and Bicknell,D.C. **(1987).** Synergistic myelopoietic actions in vivo after administration to mice of combinations of purified natural murine colony-stimulating factor I, recombinant murine interleukin 3, and recombinant murine granulocyte/macrophage colony stimulating factor. Proc.Nat.Acad. 84, **3871-3875.**

Trentin,J.J. **(1971).** Determination of bone marrow stem cell differentiation by stromal hemopoietic inductive microenvironments (HIM)[1]. Am.J.Pathol. **65, 621-8.**

Wilson,F.D. **(1984).** Hematopoietic Stroma and Microenvironment. In: "Current Methodology in Experimental Hematology", (S.J.Baum, Ed.), pp. **210-292.** S. Karger,Basel.

Zucali,J.R., Dinarello,C.A., Oblon,D.J., Gross,M.A., Anderson,L., and Weiner,R.S. **(1986).** Interleukin 1 stimulates fibroblasts to produce granulocyte-macrophage colony-stimulating activity and prostaglandin E. J.Clin.Invest. 77, **1857-1863.**

#2 HD Cells of Blood.

Schalm,O.W. **(1965).** **The** Leukemia Complex. In Veterinary Hematology, p **249.** 2nd Ed. Lea and Febiger, Philadelphia.

Archer,G.T., Air,G., Jackas,M., and Morell,D.B. **(1965).** Studies on rat eosinophil peroxidase. Biochim.Biophys.Acta 99, **96-101.**

Bessis,M. **(1972).** The Granulocytic Series. In "Living Blood Cells land their Ultra Structure", p. **318.** Springer-Verlag, New York.

Cronkite,E.P., **(1969).** Kinetics of granulocytopoiesis. Nat. Cancer Inst. Monograph 30, **51-62.**

Spitznagel,J.K., Dalldorf,F.G., Leffell,M.S., Folds,J.D., Welsh,I.R., Cooney,M.H., and Martin,L.E. **(1974).** Character of azurophil and specific granules purified from human polymorphonuclear leukocytes. Lab. Invest. 30, 774-785.

Stein,R.B. **(1984).** Granulocytosis and granulocytic leukemoid reactions. In "Laboratory Hematology", (J.A. Koepke, ed.), pp. **153-187.** Churchill Livingstone, New York.

Snipes,M.B., Boecker,b.B. and McClellan,R.O. **(1983).** Retention of monodisperse or polydisperse aluminosilicate particles inhaled by dogs, rats and mice. Toxicol. Appl. Pharamcol. 69, **345-362.**

Warner,A.E., and Brain,J.D. **(1986).** Intravascular pulmonary macrophages: A novel cell removes particles from blood. Am. J. Physiol. 250, **728-732.**

Bloom,A.L., and Thomas,D.P. **(1981).** Haemostasis **and** Thrombosis. Pp. **77-78.** Churchill Livingstone, Edinburgh.

Chesterman,C.N., Gallus,A.S., and Pennington,D.G. **(1982).** Platelets, the vessel wall and antiplatelet drugs. In "Recent Advances in Haematology", **34d** ed., pp. **253-284,** Edinburgh, Churchill Livingstone.

Handagama,P.J., and Feldman,B.F. **(1986).** Drug Induced Thrombocytopenia. Vet.Res.Comm. **10, 1-20.**

Nakeff,A. **(1984).** Megakaryocytic Cells. In "Current Methodology in Experimental Hematology", (S.J. Baum, Ed.), pp. **131-209.** S. Karger, Basel.

#1 HD Xenobiotic Metabolism

Bond,J.A., Dahl,A.R., Henderson,R.F., and Birnbaum,L.S. **(1987).** Species differences in the distribution of inhaled butadience in tissues. Am. Ind. Hyg.Assoc.J. **48, 867-872.**

Cavalieri,E., and Rogan,E. **(1985).** Role of radical cations in aromatic hydrocarbon carcinogenesis. Env. Hlth. Perso. **64, 69-84.**

Eastmond,D.A., Smith,M.T., and Irons, R.D. **(1987).** An interaction of benzene metabolites reproduces the myelotoxicity observed with benzene exposure. Toxicol.Appl.Pharmacol. 91, **85-95.**

Glazko,A.J., Wolff,L.M., Dill,W.A., and Bratton,A.C. **(1949).** Biochemical studies on chloramphenicol (chloromycetin) II. Tissue distribution and excretion studies. J. Pharmacol. Exp. Ther. 96, **445-459.**

Gonasun,L.M., Witmer,C., Kocsis,J.J., and Snyder,R. **(1973).** Benzene metabolism in mouse liver microsomes. Toxicol.Appl.Pharmacol. 26, **398-406.**

Haseman,J.K., Crawford,D.D., Huff,J.E., Boorman,G.A., and McConnell,E.E. **(1984).** Results from **86** two-year carcinogenicity studies conducted by the National Toxicology Program. J. Toxicol. Environ. Hlth. 14, **621-639.**

Hill,D.L., Laster,W.R., and Struck,R.F. **(1972).** Enzymatic metabolism of cyclophosphamide and nicotine and production of a toxic cyclophosphamide metabolite. Cancer Res. 32, **658-665.**

Irons,R.D. **(1985).** Quinones as toxic metabolites of benzene. J. Toxicol.Environ.Hlth. 16, **673-678.**

Morrison,M., and Allen,P.A. (1966). lacatoperoxidase: Identification and isolation from the harderian and lacrimal glands. Science 152, 1626-1628.

Osborne,J.C., Metzler,M., and Neumann,H.-G. (1980). Peroxidase activity in the rat zymbal gland and its possible role in the metabolic activation of aminostilbenes in the target tissue. Cancer Lett. 8, 221-226.

Reddy,B.G., Pohl,L.R., and Krishna,G. (1976). The requirement of the gut flora in nitro-benzene induced methemoglobinemia in rats. Biochem.Pharmacol. 25, 1119-1122.

Rickert,D.E. (1985). Mammalian and bacterial metabolism of nitroaromatic compounds. In "Toxicity of Nitroaromatic compounds" (Rickert,D.E. ed). pp 87-102, Hemisphere, New York.

Sladdek,N.E. (1972). Therapeutic efficacy of cyclophosphamide as a function of its metabolism. Cancer Res 32, 535-542.

Tanaka,A., and Watanabe, M. (1982). Distribution and metabolism of l-Propyl-l-nitrosourea in rats. Cancer Res. *42*, 3837-3842.

Witz,G., Rao,G.S., and Goldstein,B.D. (1985). Short term toxicity of trans, transmuconaldehyde. Toxicol.Appl.Pharmacol. 80, 511-516.

Yunis,A.A., Miller,A.M., Salem,Z.,Corbett,M.D., and Arimura,G.D. (1980). Nitroso-chloarmphenicol: possible mediator in chloramphenicol-induced aplsatic anemia. J.Lab.Clin.Med. 96(1): 36-46.

#1 HD Mechanisms of Toxicity

Alwmark,A., Bengmark,S., Gullstrand,P., and Zoucas,E. (1986). Hypersplenism-effect on haemostasis. An experimental study in the rat. Res. Exo. Med. 186(1), 21-27.

Beutler,E. (1985). Chemical toxicity of the erythrocyte. In "Toxicology of the Blood and Bone Msarrow" (R.D. Irons, ed.) , pp. 393-40. Raven Press, New York.

Bono,V.H. Jr. (1976). Review of mechanisms of action studies *of* the nitrosoureas. Cancer Treatment Reoorts 60, 699-702.

Cines,D.B., and Schreiber,A.D. (1979). Effect of anti-$P1^{A1}$ antibody on human platelets. I. The role of complement. Blood 53, 567-577.

Fries,L.F., and Frank,M.M. (1987). Molecular mechanisms of complement action. In "Molecular Basis of Blood Diseases" (Stamatoyanuopoulos,G., Neihuis,A.W., Leder,P., and Majerus,P.W. eds.), pp 450-498. W.B. Saunders, Phi1adelphia,PA.

Gangarosa,E.J., Johnson,T.R., and Ramos,H.S. (1960). Ristocetin-induced thrombocytopenia: Site and mechanism of action. Arch. Intern.Med. 105, 83-89.

Jagus,R., and Kay,J.E> (1979). Distribution of lymphocyte messenter RNA during stimulation by phytohaemagglutinin. Eur.J.Biochem. 100(2), 503-510.

Pfeifer,R.W., and Irons,R.D. (1982). Effect of benzene metabolite on PHA-stimulated lymphopoiesis in rat bone marrow. J. Reticuloendothelial Soc. 31, 155-170.

Smith,R.P. (1986). Toxic Responses of the Blood. In "Casarett and Doull's Toxicology", 3rd edition, (C.D. Klaassen, M.O. Amdur, and J. Doull, eds.), pp 223-244. Macmillan Publishing Company, New York.

Wierda,D., Irons,R.D., and Greenlee,W.F. (1981). Immunotoxicity in C57BL/6 mice exposed to benzene and Araoclor 1254. Toxicol. and Applied Pharmacol. 60, 410-417.

#2 HD Bone Marrow Suppression.

Byrne,P., Tew,K., Jemionek,J., MacVittie,T., Erickson,L., and Schein,P. (1984). Cellular and molecular mechanisms of the bone marrow sparing effects of the glucose chloroethylnitrosourea, Chlorozotocin. Blood **63,** 759-767.

Darzynkiewicz,Z., Sharpless,T., Staiano-Coico,L., and Melamed,M.R. (1980). Subcompartments of the GI phase cell cycle detected by flow cytometry. Proc.Nat.Acad.Sci.USA **77,** 6696-6699.

Gaido,K., and Wierda,D. (1985). Modulation of stromal cell function in DBA/2J and B6C3F1 mice exposed to benzene or phenol. Toxicol.Appl.Pharmacol. 81, 469-475.

Harris,R.A., and Pennington,D.G. (1984). The effects of low-dose vincristine on megakaryocytes colony-forming cells and megakaryocyte ploidy. Br.J.Haematol. **57,** 37-48.

Hoagland,H.C. (1982). Hematologic complications of cancer chemotherapy. Seminars in Oncology 9, 95-102.

Hoshi,J., and Weiss,L. (1978). Rabbit bone marrow after administration of saponin. Lab.Invest. 38, 67-80.

Howard,A., and Pelc,S.R. (1951). Nuclear incorporation of phosphorus^{32} as demonstrated by autoradiographs. Exo.Cell Res. 2, 178-187.

Irons,R.D., Heck,H.d'A., Moore,B.J., and Muirhead,K. (1979). Effects of oshort term benzene administration on bone marrow cell cycle kinetics in the rat. Toxicol.Appl.Pharmacol, 51, 399-409.

Irons,R.D., and Neptun,D.A. (1980). Effects of the principle hydroxy metabolites of benzene on microtubule polymerization. Arch.Toxicol. 45, 297-305.

Irons,R.D., Neptun,D.A., andn Pfeifer,R.W. (1981). Inhibition of lymphocyte transformation and microtubule assembly by quinone metabolites of benzene: Evidence for a common mechanism. J. Reticuloendothlelial Soc. 30, 359-372.

Irons,R.D., Pfiefer,R.W., Aune,T.M., ana Pierce,C.W. (1984). Soluble immune response suppressor inhibits microtubule function in vivo and microtubule assembly in vitro. J.Immunol. 133, 2032-2036.

Irons,R.D., Smith,C.N., Stillman,W.S., Shah,R.S., Steinhagen,W.H., and Leiderman,L.J. (1986a). Macrocytic-megaloblastic anemia in male NIH swiss mice following repeated exposure to 1,3-butadience. Toxicol.Appl.Pharmacol. 85, 450-455.

Kalf,G.F., Rushmore,T., and Snyder,R. (1982). Benzen inhibits RNa synthesis in mitochondria from liver and bone marrow. Chem.Bio.Interact. 42, 353-370.

Mitogens. Biochem.Soc.Transactions. p. 288.

King,A.G., Landreth,K.S., and Wierda,D. (1988). Bone marrow stomal cell regulation of B-lymphophopoiesis. Ii. Mech. of hydroquinone inhibition of pre-B cell maturation. (In press).

MacGregor,J.T., Sehr,C.M., and Gould,D.H. (1980). Clastogen-induced micronuclei in peripheral blood erythrocytes: The basis of an improved micronucleus test. Environ.Mutagen. 2, 509-514.

Marsh,J.C. (1985). Chemical Toxicity of the Granulocyte. In "Toxicology of the Blood and Bone Marrow" (Irons,R.D. Ed.), p 55. Raven Press, New York.

Santesson,C.G. (1897). Chronic poisoning with coal tar benzene: Four deaths. Clinical and pathological-anatomical observations of several colleagues and illustrating animal experiments. Arch.Hyg. (Munich) 31, 336-376.

Seyama,T., Kajitani,T., Inoh,A., Yanagihara,K., Kamiya,K., and Yokoro,K. (1985). Synergistic effect of radiation and N-nitrosoethylurea in induction of lymphoma in mice: cellular kinetics and cardinogenesis. Jpn.J.Cancer Res. (Gann) 76, 20-27.

Swenberg,J. (1976). Chemical induction of brain tumors. In "Advances in Neurology 15" (R.a. Thompson and J.R. Green, eds.), pp 85-99. Raven Press, New York.

Testa,N.G., Xy,C.X., and Hendry,J.H. Manuscrsipt in preparation. 1984.

Thomas,D.J., Reasor,M.J., and Wierda,D. (1988). macrophage regulation of myelopoiesis isi altered by exposure eto the benzene metabolite hydroquinone. (In press). Toxicol. and Applied Pharmacology.

Trainor,K.J., and Morley,A.A. (1976). Screening of cytotoxic drugs for residual bone marrow damage. J.Nat.Canc.Inst. 57, 1237-39.

van PuttenL.M., and Lelieveld,P. (1971). Factors determining cell killing by chemotherapeutic agents in vivo. Ii. Melphalan, chlorambucil and nitrogen mustard. Europ.J.Cancer 7, 11-16.

#2 HD Cytopenias.

Abrahamsen,A.F., Klepp,O., Fossa,S.>D., and Sonstevold,,A. (1986). Transienet vinblastine-induced thrombocytopenia during chemotherapy with vinblastine, cis-platinum and bleomycin. Scand.J.Haematol. 37, 44-49.

Davis,W.M. (1984). Hapten-induced, immune-mediated thrombocytopenia in a dog. J.Am.Vet.Assoc. 184, 976-967.

Karnofsky,D.A. (1968). Cancer chemotherapeutic agents. CA 18(2), 72-81.

Petursson,S.R., and Chervenick,P.A. (1982). megakaryocytopoiesis and granulopoiesis following cyclophosphamide. J.Lab.Clin.Med. 100, 682-694.

Selling,L. (1916). Benzol as a leucotoxin. Studies on the degeneration and regeneration of the blood and hematopoietic organs. Johns Hopokins Hosp.Rep 17, 83-142.

Snyder,C.A., Goldstein,B.D., Sellakumar,A.R., Bromberg,I., Laskin,S., and Albert,R.E. (1980). The inhalation toxicology of benzene: Incidence of hematopoietic neoplasms and hematotoxicity in AKR/J and C57BL/6 Mice. Toxicol. and Applied Pharmacol. 54,323-331.

#2 HD Aplastic Anemia

Adamson,J.W. (1985). Neoplasms of the hematopoietic stem cell. Prog.Clin.Biol.Res. 184, 393-402.

Boggs,D.R., and Boggs,S.S. (1976). The pathogenesis of aplastic anemia: A defective pluripotent hematopoietic stem cell with inappropriate balance of differentiation and selef-replication. Blood. 48(1), 71-76.

Boggs,D.R., Boggs,S.S., Chervenick,P.A., and Patrene,K.D. (1980). Murine recovery from busalfan-induced hematopoietic toxicity as assessed by three assays for colony-forming cells. Am.J.Haematol. 8, 43-54.

Cronkite,E.P., Iinoue,T., Carsten,A.L., Miller,M.E., Bullis,J.E., and drew,R.T. (1982b). Effects of benzene inhalation on murine pluripotent stem cells. J.Toxicol.Environm.Health 9, 411-421.

denOttolander,G.J., Tevelde,J., Veenhof,W., Klieverda,K., Haak,H.L., and Spander,P.J. (1982). Busulfan aplasia in rabbits: a mode for human aplastic anemia. Br.J.Haematol. 51, 265-276.

Fleidner,T.M., Flad,H.-D., Bruch,C., Calvo,W., Goldmann,S.F., Gerbst,E., Hugl,E., Huget,R., Korbling,M., Krumbacher,K., Nothdurft,W., Ross,W.M., Schnappauf,H.-P., and Steinbach, I. **(1976).** Treatment of aplastic anemia by blood stem cell transfusion: A canine model. Haematologica 61(2), **141-156.**

Gordon-Smi'th,E.C., Malkovska,V., Myint,A.A., Gibson,F., and Milne,A. **(1988).** Growth factors and lymphokines in aplastic anemia. Exp.Hematology 27a, **419.**

Haake,H.L. **(1980).** Experimental drug-induced aplastic anaemia. Clinics in Haematol. 9, **621-639.**

Harigaya,K., Miller,M.E., Cronkite,E.P., and Drew,R.T. **(1981).** The detection of in vivo hematotoxicity of benzene in vitro liquid bone marrow cultures. Toxicol and Applied Pharmacol 60, **346-353.**

Morley,A., and Blake,J. **(1974a).** An animal model of chronic aplastic marrow failure. I. Late marrow failure after busulfan. Blood 44, **49-56.**

Morley,A., and Blake,J. **(1974b).** Haemopoietic precursor cells in experimental hypoplastic marrow failure. Australian J.Exp.Biol.Med.Sci. 52, **909-914.**

Morley,A., Trainor,K., and Blake,J. **(1975).** A primary stem cell lesion in experimental chronic hypoplastic marrow failure. Blood 45, **681-688.**

Seidel,H.-J. **(1972).** Pancytopenia in CBA mice after Rauscher virus infection. J.Nat.CancerInst. 48, **959-964.**

Uyeki,E.M., Ashkar,A.E., Shoeman,D.W., and Bisel,T.U. **(1977).** Acute toxicity of benzene inhalation to hemopoietic precursor cells. Toxicol. and Applied Pharmacol. 40, **49-57.**

Williams,D.M., Lynch,R.E., and Cartwright,G.E. **(1973).** Drug-induced aplastici anemia. Seminars in Hematol. 10, **195-223.**

Zoumbos,N.C., Gasson,P., Djeu,J.Y., and Yong,N.S. **(1985).** Interferon is a mediator of hematopoietic suppression in aplastic anemia in vitro and possibly in vivo. Proc. Nat. Acad.Sci. **82, 188-192.**

#2 HD **Myelodysplasia Syndrome.**

Auger,M.J., Nash,J.R.G., Mackie,M.J. **(1986).** Marrow involvement with **T** cell lymphoma initially presenting as abnormal myelopoiesis. J.Clin.Pathol. **39, 134-137.**

Bagby,G.C. Jr. **(1986).** The concept of preluekemia: Clinical and laboratory studies. CRC Crit.Rev.Oncol./Hematol. 4, **203-220.**

Baumann,M.A., Libnoch,J.A., Hansen,R.M., Heckman,M.G., and Hanson,G.A. **(1985).** Concurrent myelodysplasia and lymphoproliferation: a disorder of the true pluripoential stem cell? Quart.J.Med. New Series **55,** 218, **199-211.**

Berneman,Z.N., Van Bock Staele,D., DeMeyer,P., van der Planken,M., Vertessen,F., DeBock,R.,, Peetermans,M.E. (1986). A myelodysplastic syndrome preceding acute 1 ymphoblastic leukemia. Br.J.Haematol. 60, 353-354.

Block,M., Jacobson,L.O., and Bethard,W.F. (1953). Preleukemic acute human 1eukemia. J.Am.Med.Assoc. 152, 1018-1028.

Camba,L., and Joyner,M.V. (1985). Refractory anaemia terminating in a combined lymphoproliferative and myeloproliferative disorder. J.Clin.Pathol. 38, 297-300.

Fohlmeister,I., Schaefer,H.E., and Fischer,R. (1982). On the pathogenesis of preleukemic myelodysplastic syndromes. J.Cancer Res. 104, 249-261.

Fritz,T.E., Tolle,D.V., and Seed,T.M. (1985). The preleukemic syndrome in radiation-induced myelogenous leukemia and related myeloproliferative disorders In "The Preleukemic Syndrome (Hemopoietic dysplasia)" (Bagby,G.C. ed.). pp 87-100,CRC Press, Boca Raton,FL.

Galton,D.A.G. (1986). The myelodysplastic syndromes. Scand.J.Haematol. 36, 11-20.

Greenberg,P.L. (1986a). Biologic abnormalities in the myelodysplastic syndromes and myeloproliferative disorders. Acta Haematol.Jpn. 49, 1509-1527.

Greenberg,P.L. (1986b). In vitro culture techniques defining biological abnormalities in the myelodysplastic syndromes and myeloproliferative disorders. Clinics in Haematol. 15(4), 973-993.

Irons,R.D., and Cathro,H.P. (1988). Potential in fluence of dose and regimen on benzene-induced myelodysplastic syndrome and 1eukemia in C57BL/6 mice. Environ.Hlth.Persp. In press.

Irons,R.D., Cathro,H.P., Stillman,W.S., Steinhagen,W.H., and Shah,R.S. (1988). Susceptibility to 1,3-Butadiene-induced leukemogenesis correlates with endogenous ecotropicretroviral background in the mouse. Cancer Res. (In submission).

Levine,E.G., and Bloomfield,C.D. (1986). Secondary myelodysplastic syndromes and leukemias. Clinics in Haematoloav 15, 1037-1079.

Tolle,D.V., Seed,J.M., Cullen,S.M., Fritz,T.E., Poole,C.M., and Lombard,L.S. (1983). The preleukemic syndrome in radiation-induced canine myeloid leukemia and related myeloproliferative diseases. Exp.Hematol. 11 (Suppl. 141, 154.

#2 HD Leukemias

Maloney,W.C., Boschetti,A.E., and King,V.P. (1969). Observations on leukemia in Wistar Furth rats. Cancer Res. 29, 938-946.

Maloney,W.C., Boschetti,A.E., and King,V.P. (1970). Spontaneous leukemia in Fischer rats. Cancer Res. 30, 41-43.

McCulloch,E.A., Smith,L.J., and Minden,M.D. (1982). Normal and malignant haemopoietic clones in man. Cancer Surveys 1, 280-298.

Pattengale,R.K., and Taylor,C.R. (1981). Immunomorphologic classification of murine lymphomas and related leukemias. Proceedings of the Rodent Lymphoma Workshop, pp. 22-23, National Center for Toxicologic Research Press, jefferson AK.

Pattengale,R.K., and Taylor,C.R. (1983). Experimental models of lymphoproliferative disease. The mouse as a model for human non-Hodgkin's lymphomas and related leukemias. A.J. Pathol. 113, 237-265.

Rao,G.N., Birnbaum,L.S., Collins,J.J., Tennant,R.W., and Skow,L.C. (1988). Mouse strains for chemical carcinogenicity studies: Overview of a workshop. Fund. and Appl. Toxicol. 10, 385-394.

Shay,H., Gruenstein,M., and Harris,C. (1955). Experimental Leukemias in the rat. Acta. Haematol. 14, 337-353.

Siegler,R., and Rich,M.A. (1966). Pathogenesis of murine leukemia. Nat.Cancer Inst. Monograph *22*, pp 525-547.

Siegler,R., and Rich,M.A. (1967). Pathogenesis of virus-induced myeloid leukemia in mice. J.Nat.Cancer Inst. **38**, 31-50.

Simonds,J.P. (1925). Leukemia, pseudoleukemia and related conditions in the Slye stock of mice. J. Cancer Res. 9, 329-373.

STromberg,P.C. and Vogtsberger,L.M. (1983). Pathology of the mononuclear cell leukemia of Fischer rats. I. Morphologic Studies. Vet.Pathol. 20, 698-708.

Stromberg,P.C., Rojko,J.L., Vogesberger,L.M., Cheney,C., and Berman,R. (1983). Immunologic biochemical and ultrastructural characterization of the leukemia cell in F344 rats. J.Nat.Cancer Inst. 71, 173-181.

Irons,R.D., Stillman,W.S., Shah,R.S., Morris,M.S., and Higuchi,M. (1986b). Phenotypic characterization of 1,3-butadiene-induced thymic lymphoma in male B6C3F1 mice. The Toxicologist 6, 21.

Gross,L. (1970). "Oncogenic viruses", 2nd ed., pp. 286-452, Pergamon Press, New York.

Huff,J.E., Melnick,R.L., Solleveld,H.A., Haseman,J.K.., Powers, M., and Miller,R.A. (1985). Multiple organ carcinogenecity of 1,3-butadiene in B6C3F1 mice after 60 weeks of inhalation expsoure. Science 227, 548-549.

Goodman,D.G., Ward,J.M., Squire,R.A., Chu,K.C., and Linhart,M.S. (1979). Neoplastic and non-neoplastic lesions in aging F344 rats. Toxicol. and Appl. Pharmacol. **48**, 237-248.

Goodman,D.G., Bates,R.R., Ward,J.M., Frith,C.H., and Sauer,R.M. (1981). Common 1esions in aged B6C3F2 and BALV/cStCrifC3H/Nctr mice. Armed Forces Institute of Pathology, pp 15-19.

Cloyd,M.W., and Evans,L.H. (1987). Endogenous retroviral env expression in primary murine leukemias: lack of xenotropic antigens but presencse of distinct mink cell focus-forming env subtypes correlating with ecotropic virus inoculated and mouse strain. J.Nat.Cancer Inst. 78, 181-189.

Coltman,C.A. (1982). "Adult Leukemias 1" pp 61-208. Martinus Niijhoff, Boston.

Cronkite,E.P. (1987). Chemical Leukemogenesis: Benzene as a Model. Sem. in Hematol. 24, No. 1, 2-11.

Dunn,T.B. (1954). Normal and pathologic anatomy of othe reticular tissue in laboratory mice, with a classification and discussion of neoplasms. J.Nat.Cancer Inst. 14(6), 1281-1433.

Endicott,K.M. (1980). Re-evaluation of the pathology findings of studies on nitrite and cancer. Histologic lesions in Sprague-Dawley rats. Dept. of Health and Human Services, Food and Drug Administration.

Fialkow,P.J., Jacobson,R.J., and Papayannopoulou, T. (1977). Chronic myelocytic leukemia: Clonal origin in a stem cell common to the granulocyte, erythrocyte, platelet and monocyte-macrophage. Am.J.Med. 63, 125-131.

Fialkow,P.J., Denman,A.M., Singer,J., Jacobson,R.J., and Lowenthal,M.N. (19788b). In "Differentiation of Normal and Neoplastic Hematopoietic Cells. (B. Clarkson,P.A. Marks, and J.E.Till, eds.), pp 131-144. Cold Spring Harbor Laboratory, Vol. 5.

Fialkow,P.J. (1980). Clonal and stem cell origin of blood clel neoplasms. In "Contemporary Hematology/Oncology" (LoBue,J. ed.). pp 1-46, Plenum Press, New York.

Fialkow,P.J., Singer,J.W., Adamson,J.W., Vaidya,K., Dow, L.W., Ochs,J., and Moohr,J.W. (1981). Acute nonlymphocytic leukemia: heterogeneity of stem cell origin. Blood 57, 1068-1073.

Barnes,W.A., and Sisman,I.E. (1939). Myeloid leukemia and non-malignant extramedullary myelopoiesis in mice. Am.J.Cancer 37(1), 1-35.

Wintrobe,M.M. (1974). Clinical Hematology. (Lee,G.R., Boggs,D.R., Bithell,T.C., Athens,J.W., and Foerster,J. eds.), 7th ed., pp 1431. Lea & Febiger, Phil., Pa.

Crouse,D.A., Jordan,R.K., and Sharp,J.G. (1984). Thymic Microenvironment. In "Current Methodology in Experimental Hematology' (S.J. Baum, Ed.). pp 293-230, S. Karger ,Basel .

Eaves,C.J., Krystal,G., and Eaves,A.C. (1984) Erythropoietic Cells. In "Current Methodology in Experimental Hematology" (S.J. Baum,Ed.), pp 81-111. S. Karger, Basel.

Fruhman,G.J. (1964). Cell counts of mouse bone marrow. Life Sci. 3:737-48, Jul 64.

Harris,E.B. (1957). In vivo uptake of radioactive iron by the erythroid cells of rat bone marrow. In "Acvances in Radiobiology" (Hevesy et al? eds.). p. 26, Chas.C. Thomas, Springfield.

Irons,R.D. and Stillman,W.S. (1985). Flow Cytofluorometric Analysis of Blood and Bone Marrow. In "Toxicology of the Blood and Bone Marrow". (Richard D. Irons, ed.), pp 101-118. Raven Press, New York.

Johnson,G.R. (1984). Clonal assays for multipotential hemopoietic cells in vitro. In "Current Method in Experimental Hematology" (S.J. Baum, ed.), pp. 63-80, karger, Basel 1984.

Keller,G.M., and Phillips,R.A. (1982). Detection in vitro of a unique multipotent hematopoietic progenitor. J.Cell Phvsiol.Suppl.I, 31-36.

Melamed,M.R., Mullaney,P.F., and Mendelsohn,M.L. (1979). Flow Cytometry and Sorting. John Wiley and Sons, New York.

Mulder (1974). Mulder, E., Counting cells in human bone marrow. Am.J.Clin.Pathol. 61: 199-202, Feb. 74.

Smith,C.N., Neptun,D.A., andn Irons,R.D. (1986). Effect of sampling site and collection method on variations in baseline clinical pathology. 11. Hematology. Fund. and Appl.Toxicol. 7, 658-663.

Spooncer,E., and Dexter,T.M. (1984). Long-term Bone Marrow Cultures. In "Current Methodology in Experimental Hematoogy" (S.J. Baum,ed.), pp. 366-383. S. Karger, Basel.

Ward,H.P., Tauxe,W.N., and Kiely,J.M. Jr. (1966) ^{59}Fe autoradiography of bone marrow culture from normal subjects and patients with uraemia and pernicious anaemia. Br.J.haematol. 12, 99-104.

Youse,J.H., and Barry,W.E. (1973). Quantitation of reticuloendothelial cells in rat marrow. J. Reticuloendothel. Soc. 14(3), 258-265.

Zucali,J.R., Dinarello,C.A., Oblon,D.J., Gross,M.A., Anderson,L., and Weiner,R.S. (1986). interleukin 1 stimulates fibroblasts to produce granulocyte-macrophage colony-stimulating activity and prostaglandin E. J. Clin.Invest. 77, 1857-1863.

Table 1

Hematopoietic Growth Factors

Growth Factor	Synonyms	Source	Target Cells - Known Activity
IL-3	Multi-CSF	T-lymphocytes	Supports growth of early progenitor cells: CFUs, CFC-Mix, pre-lymphocytes, macrophage progenitors and mast cells. Acts synergistically with Epo or G-CSF to stimulate BFU-E and CFU-G.
M-CSF	CSF-1	Endothelial cells, macrophages Mouse L cells	Supports growth of macrophage progenitors
M-CSF	CSF-2	Endothelial Macrophages lung cells	Supports macrophage and granulocyte progenitors. Acts synergistically with other factors to support BFU-E, CFU-G and CFU-Meg.
G-CSF		T lymphocytes macrophages	Stimulates CFU-G. Acts synergistically with IL-3 and GM-CSF to support CFU- and CFU-M.
Erythro-poietin		Kidney	Stimulates CFU-E and proerythroblasts.

Figure 1. Schematic diagram of hematopoiesis, showing the relationship between proliferation and self-renewal in the stem cell and precursor cell compartments.

Figure 2. Kinetics of granulocyte formation illustrating the relatively small proportion of granulocyte pool in circulating and marginated compartments (Cronkite, 1969).

Figure 3. (a) DNA cell cycle based on hypothetical 24 hr. transit time. (b) Phases of the cell cycle as defined by relative DNA content of cells.

Figure 4. DNA cell cyle histogram obtained by flow cytofluorernetric analysis of asynchronously dividing rabbit bone marrow cells. (Irons and Stillman, 1985).

Figure 5. (a) Photomicrograph of normal rabbit bone marrow showing normal cellularity. (b) Hypoplastic bone marrow following 10 days repeated benzene exposure. Note vertical absence of viable precursor cells. (Methacrylate: Toluidine Blue).