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ANALYSIS OF HEMATOPOIETIC PROGENITOR CELL CYCLE CONTROL
IN THE MYELOPROLIFERATIVE DISORDERS

by

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ABSTRACT

The myeloproliferative disorders (MPD) comprise an interesting group of hematological neoplasms in which clonal expansion is initiated at the level of the pluripotent stem cell compartment but differentiation proceeds essentially normally. Although evidence for the involvement of specific genetic changes exist in one of these diseases (chronic myeloid leukemia, CML), the nature of the lesion that permits the progeny of a single stem cell to dominate the mature cell compartment has not been elucidated. Application of clonal assay systems to the study of the MPD has provided information about the numbers, proliferative capacity, physical properties and the responsiveness to regulatory factors of hemopoietic progenitors from all cell lineages. However, clonal assays can offer only limited information about the processes that underly stem cell regulation. Some of the limitations imposed by these assays may be overcome by the use of long-term cultures in which primitive and pluripotent progenitors may be maintained for at least 2 months.

The purpose of this thesis was to determine if consistent alterations in cell cycle activity were characteristic of progenitors in MPD patients, and to evaluate the potential of the long-term culture system for further investigations of any changes observed. The proliferative behaviour of clonogenic progenitors from the blood and bone marrow of a large number of MPD patients was compared with that of normal individuals using the ^3H -thymidine cell suicide technique. These experiments showed that all progenitor classes in the blood and marrow of patients with CML and polycythemia vera (PV), which in normal individuals are quiescent, had a significant component of cycling cells. In addition, a consistent association of this abnormality in cycling control with expression of erythropoietin (EP)-independence in patients with essential thrombocytosis (ET) was revealed. Further studies were then undertaken to

determine if these abnormalities could be reproduced in vitro. Experiments with normal marrow showed that the most primitive progenitor classes located in the adherent fraction of standard long-term cultures undergo cyclic changes of proliferative activity with each weekly addition of new growth medium. These studies suggested that the proliferative activity of normal primitive hemopoietic cells may be both positively and negatively regulated by close range interactions with marrow stromal elements. In contrast, in similar experiments with long-term cultures established with PV marrow, where maintenance of neoplastic cells could be documented, analogous primitive progenitor cells in the adherent layer failed to return to a quiescent state and remained continuously in cycle.

From previous experiments with CML patients, it was already known that Ph¹-positive progenitors usually disappeared rapidly in long-term cultures established with CML marrow. Therefore, as an alternative approach CML peripheral blood cells were seeded onto preestablished normal marrow adherent layers, since preliminary studies had suggested that this would allow sufficient numbers of primitive Ph¹-positive progenitors to be maintained for cycling studies. Analysis of such cultures together with studies of appropriate normal controls, revealed the same lack of cycling regulation in CML as previously shown for PV. In addition, studies of control cultures showed that in the absence of an adherent layer, normal peripheral blood progenitors cycle continuously, again suggesting that one regulatory function of the adherent layer is to maintain normal progenitors in a quiescent state.

These studies demonstrate that consistent abnormalities of cell cycle control characterize the primitive progenitor compartments in MPD patients, and that these abnormalities can be reproduced in vitro. Initial findings with the long-term marrow system suggest that these abnormalities may be due to the insensitivity of primitive neoplastic cell types to respond to factors that arrest their normal counterparts from further progression through the cell cycle.

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LIST OF ABBREVIATIONS

| | |
|-----------------|--|
| MPD | myeloproliferative disorders |
| CML | chronic myelogenous leukemia |
| PV | polycythemia vera |
| ET | essential thrombocytosis |
| Ep | erythropoietin, a glycoprotein required for <u>in vitro</u> and <u>in vivo</u> erythropoiesis |
| Ph ¹ | Philadelphia chromosome |
| CFU-S | spleen colony-forming unit, pluripotent stem cells capable of macroscopic spleen colony formation in irradiated mice |
| CFU-C | culture colony-forming unit, granulopoietic progenitors that generate colonies of >20 granulocytes and/or macrophages in culture |
| CFU-E | erythroid colony-forming unit, relatively late erythropoietic progenitor cells that generate single or double clusters of 8-50 erythroblasts in culture |
| BFU-E | erythroid "burst"-forming unit, erythropoietic progenitors more primitive than CFU-E, that generate colonies containing multiple erythroblast clusters in culture (mature BFU-E: 3-8 clusters, Primitive BFU-E:>8 or >16 clusters) |
| CFU-M | megakaryocyte colony-forming unit, progenitors that generate colonies of >2 megakaryocytes |
| CFU-GEMM | mixed colony-forming unit, progenitors that generate colonies of granulocytes, erythrocytes, macrophages, and megakaryocytes |
| CSF | colony stimulating factor (also referred to as colony stimulating activity, CSA) a family of glycoprotein molecules required for <u>in vitro</u> granulocyte/macrophage colony growth |
| IL-3 | interleukin-3, glycoprotein molecule that stimulates all types of early clonogenic myloid precursors |
| BPA | burst-promoting activity (probably identical to interleukin-3) |
| PGE | prostaglandin E |
| PDGF | platelet derived growth factor |
| ³ H | tritium |
| GAG | glycosaminoglycans, components of the extracellular matrix |

| | |
|---------|---------------------------------------|
| G6PD | glucose-6-phosphate dehydrogenase |
| DNA | deoxyribonucleic acid |
| S-phase | DNA synthetic phase of the cell cycle |
| LCM | leukocyte-conditioned medium |
| PHA | phytohemagglutinin |
| BSA | bovine serum albumin |
| FCS | fetal calf serum |
| HS | horse serum |
| PBL | peripheral blood |

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C H A P T E R I
THE HEMOPOIETIC SYSTEM

1) REGULATION OF HEMOPOIESIS

The hemopoietic system has two main components; 1) a variety of free interstitial elements composed of hemopoietic cells in various stages of maturation and; 2) a fixed stromal compartment which provides the specialized environment necessary for their maintenance and proliferation. Normal hemopoiesis is dependent on the effective interplay between these two components.

The mature blood cells - i.e the lymphocytes, granulocytes, monocytes, platelets and red blood cells - all possess specialized intracellular machinery that permit expression of their various unique biological functions. Most represent end cells that are incapable of further proliferation and are also short lived in relation to the lifespan of the individual. Thus production of new blood cells is a continuous process. The mature blood cells are maintained by the proliferative activity of less differentiated cells. The most primitive of these that are restricted in their differentiative potential retain a considerable proliferative capacity but appear to possess little capacity for self-renewal. Maintenance of their numbers is therefore thought to be dependent upon continual replenishment from a common pluripotent stem cell compartment whose members are capable of extensive self-renewal as well as differentiation down any one of the myeloid cell pathways.

The hemopoietic stroma is believed to provide both mechanical support and regulatory signals important for the growth and maintenance of blood-forming cells. However, it has only been in the last few years that

attention has focussed on the definition of the cell types involved in these processes and the development of appropriate assays and probes to analyze the mechanisms involved. Much of our present knowledge regarding the role of hemopoietic-stromal cell interactions in hemopoiesis still dates from in vivo experiments in mice. Nevertheless, the practical limitations imposed by such approaches and the difficulties in deriving analogous data in man has encouraged the development of appropriate in vitro systems. During the past twenty years a wide variety of hemopoietic colony assay systems have been developed. These permit the enumeration of various classes of hemopoietic progenitors in a given sample. Observations of the size, morphology and cellular composition of the colonies, coupled with appropriate manipulations of the culture conditions have led investigators to theorize about the functioning and regulation of various stages of hemopoiesis. By their very nature however, colony assay systems do not permit examination of the intimate contact interactions between hemopoietic and stromal cells that may be of importance in vivo. Recently, an in vitro marrow culture system has been developed which offers an opportunity to explore relationships between hemopoietic and stromal elements in a closed environment in which hemopoiesis may proceed for several weeks and which may also be manipulated. This doctoral project is concerned with the application of both types of in vitro culture systems to the study of the cell cycle control of primitive normal and neoplastic hemopoietic cells.

A. Hemopoietic Progenitor Assays

The spleen colony assay and the concept of a hemopoietic stem cell

The first application of clonal assay techniques was developed by Till and McCulloch in 1961. They injected limiting numbers of hemopoietic cells from donor mice into heavily irradiated histocompatible recipients. Within 8

to 14 days macroscopic nodules were observed on the spleens of the recipients. When examined microscopically the nodules were seen to consist of recognizable cells of the various myeloid series. Usually only a single lineage was found to predominate in each colony although, "mixed" colonies containing erythropoietic, granulocytic, and megakaryocytic cells were also found (McCulloch, 1963; Fowler et al, 1966; Chen and Schooley, 1968).

A linear relationship between the number of spleen colonies formed and the number of nucleated cells injected suggested that each colony arose from a single cell. Direct evidence for this hypothesis came with karyotypic analysis of metaphases from individual colonies generated in mice injected with mixed populations of radiation-induced chromosomally marked marrow cells. Unique karyotypes were demonstrated in 95-100% of the cells from individual colonies, therefore providing strong evidence for their single cell origin. (Becker et al, 1963; Wu et al, 1967). The entity capable of giving rise to a colony was termed a "colony forming unit-spleen" (CFU-S).

Once the clonal nature of spleen colonies had been established certain properties of CFU-S could be inferred from analysis of the cellular composition of the colonies to which they gave rise. The extensive proliferative capacity of CFU-S and its pluripotentiality was evidenced by the large size of the colonies and the presence of several cell types within individual colonies. The self-renewal ability of CFU-S was demonstrated by serial transplantation experiments in which the progeny of a single colony was injected into a secondary recipient. Production of secondary colonies comparable in size and of mixed or different cellular composition indicated that primary colonies contain cells which can also be characterized as CFU-S (Siminovitch et al, 1963; Lewis and Trobaugh, 1964; Juraskova and Tkadlecek, 1965). These properties of CFU-S - pluripotentiality, extensive

proliferative capacity and self-renewal appear to fulfill the minimum criteria for stem cells. Although considerable cytogenetic and isoenzyme studies have indicated that a common ancestral cell exists for both the myeloid and lymphoid lineages in both mouse and man (Wu et al, 1967; Abramson et al, 1972; Sacker-Walker and Hardy, 1975; Fialkow, 1978; Prchal et al, 1978; Dick et al, 1985) no direct cytological evidence exists for the presence of lymphocytes in spleen colonies.

Despite the considerable contribution that applications of the spleen colony assay have made to our knowledge of the hemopoietic system, several problems associated with its use remain unresolved. The CFU-S represent only those stem cells which develop in the spleen, and are therefore only a proportion, referred to as the "f" fraction of the total of such cells originally present in the inoculum. Not all of the cells capable of forming colonies reach the spleen (Siminovitch et al, 1963); a proportion will seed in other parts of the body, notably the bone marrow and lungs. Of the stem cells that reach the spleen, a number may be expelled (Playfair and Cole, 1965), or may not generate colonies. Though retransplantation studies have been used to estimate the f fraction, the values obtained vary widely (Siminovitch et al, 1963; Hendry, 1971). An accurate estimation of CFU-S number is made more difficult by the demonstration, using cytogenetic markers, that more than one colony may arise from a single cell (Barnes et al, 1968). Furthermore, recent studies have demonstrated that "early" spleen colonies which develop 7 to 10 days after transplantation are transient, and are composed of the progeny of a more developmentally restricted cell than the colonies which may develop a few days later (Magli et al, 1982) and cells capable of long-term hemopoietic cell repopulation may differ again from even day 14 CFU-S (Phillips et al, 1984). Finally the spleen colony assay suffers

from many of the problems that plague most in vivo assays. It is difficult to modify the culture conditions to permit examination of the effects of environmental and exogenous factors on the proliferation and differentiation of stem cells, nor can an investigator be completely aware of all the endogenous influences in the system which may affect the experimental results. Some of these problems could be overcome by cultivating hemopoietic cells in artificial media outside the body. Accordingly, much effort has been expended in attempts to develop an appropriate in vitro culture system that permits the proliferation and differentiation of the most primitive hemopoietic cells under more controlled conditions.

Clonal assay systems and the concept of committed progenitors

The concept of an intermediate compartment of hemopoietic progenitors was first suggested by in vivo studies of Ep stimulated red cell production in polycythemic mice. These experiments showed that the Ep responsive cells (ERC) detected by this assay were more primitive than the first morphologically recognizable cell, the proerythroblast, but further along the differentiation pathway than CFU-S. These "committed progenitors" were envisaged as lineage restricted, incapable of self-renewal, but still possessing a considerable proliferative capacity (Krantz and Jacobson, 1970).

Granulocyte/macrophage colony forming cells The first in vitro hemopoietic colony assay system was developed independently in the mid-sixties by two different groups (Bradley and Metcalf, 1966; Pluznick and Sachs, 1965). In both instances culture conditions were employed that permitted the formation of discrete colonies containing 50 to several hundred cells of the granulocyte/macrophage (GM) series from suspensions of mouse marrow cells. The cell of origin was named the CFU-C for colony forming unit-culture. Shortly thereafter human granulocyte/macrophage colonies were

also successfully cultured from human bone marrow (Senn et al, 1967; Pike and Robinson, 1970) and peripheral blood cells (Kurnick and Robinson, 1971) by analogous procedures (Figure 1).

Subsequently culture conditions were developed that allowed the detection of committed progenitors for all hemopoietic pathways in a variety of species including man (see review by Metcalf, 1977; Eaves and Eaves, 1984). In each instance hemopoietic cells are immobilized in a semi-solid or viscous medium composed of agar, methycellulose or plasma clot, containing essential nutrients and growth factors. Under such conditions the clonal progeny of a single precursor are held immobilized in close proximity to one another, thus permitting the enumeration of individual colonies and examination of their morphological characteristics. In such clonal assay systems the state of differentiation of the progenitor cell is usually correlated with the length of the incubation period required for the production of recognizably differentiated progeny and the size of the mature colony obtained (see review by Eaves and Eaves, 1984).

The single cell origin of all types of in vitro colonies has been established by several investigators using a variety of techniques including 1) direct microscopic observation of the formation of a colony from a single cell by replating experiments (Paran and Sachs, 1969; Johnson and Metcalf, 1977; Nakahata and Ogawa, 1982), physical isolation by plastic rings (Pluznick and Sachs, 1966), or photography (Cormack, 1976); b) isoenzyme studies of individual colonies (Prchal et al, 1976; Singer et al, 1979a) and c) mixing experiments with male and female cells and demonstration by Y chromosome analysis of the homogeneity of individual colonies (Strome et al, 1978; Fauser and Messner, 1978, Dube et al, 1981).

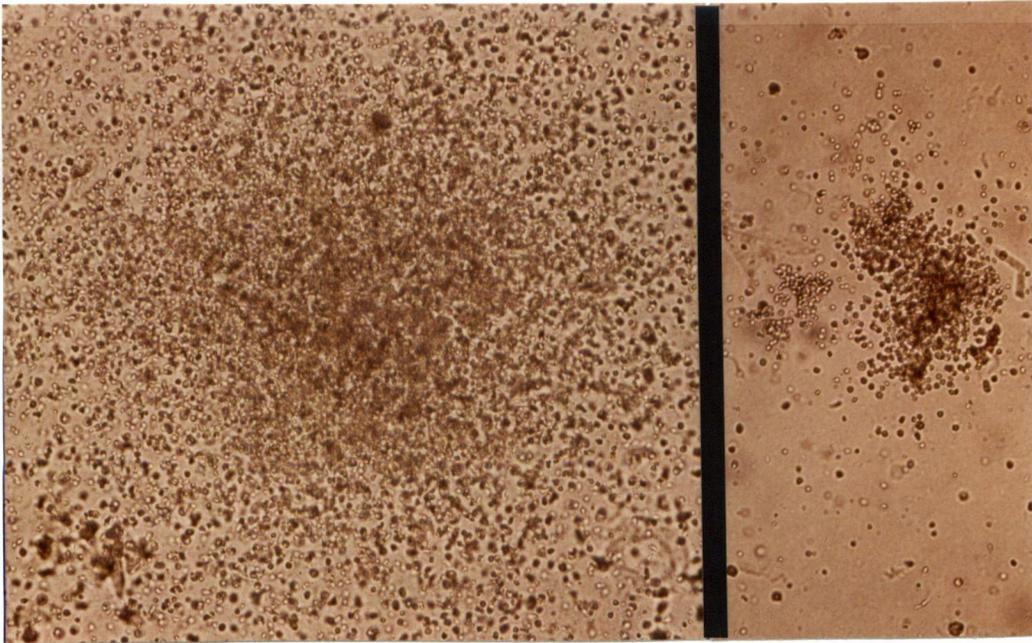
**A****B**

Figure 1. Photographs of granulocyte colonies grown in methylcellulose culture. As with erythroid colonies the differences in colony size reflects the plating of progenitors at varying stages of differentiation (X80).

A. A large granulocytic colony (>500 cells) from an early, primitive CFU-C with a high proliferative potential.

B. A small granulocytic colony (<500 cells) from a late, mature CFU-C. Such a progenitor has a low proliferative capacity and is further down the differentiation pathway than the primitive CFU-C.

Erythroid colony-forming cells Recognition of the differences in culture conditions required to support the growth of large and small erythroid colonies and demonstration of differences in the properties of their precursors (Axelrad et al, 1974, Gregory, 1976), led to the concept of a hierarchy of erythropoietic progenitor cell classes. These subpopulations of committed progenitors within the erythroid pathway could be identified by the number of clusters present in each colony and their sequential appearance in the culture dish (Figure 2). For example, the most differentiated type of erythroid colony progenitor, the colony forming unit-erythroid (CFU-E) in man yields a colony within 9 days of 1 or 2 clusters, each consisting of 8 to 64 erythroblasts. In contrast, the most primitive burst forming unit-erythroid (BFU-E) is defined in man as a progenitor capable of producing a colony of 8 or more clusters and up to 10^5 cells after 18 days of incubation (see review, Eaves and Eaves, 1984). A number of intermediate colony types composed of varying numbers of clusters and order of appearance can also be recognized (Gregory and Eaves, 1977). The number of clusters present in a burst thus provides a convenient measure of colony size which in turn appears to be predetermined by the stage of differentiation of the progenitor cell that gave rise to it. The precursor:progeny relationship of primitive BFU-E, mature BFU-E, and CFU-E has been validated in a number of ways including successive changes in responsiveness to Ep, cell volume, proliferative capacity and cell cycle activity (Axelrad et al, 1974; Gregory, 1976; Gregory and Eaves, 1978).

Megakaryocyte colony-forming cells Megakaryocyte colony forming progenitors were first detected by Nakeff and his associates (1975) using bone marrow cells from vinblastine treated mice, cultured in agar over feeder layers of mouse embryo fibroblasts. Larger colonies, containing up to 80

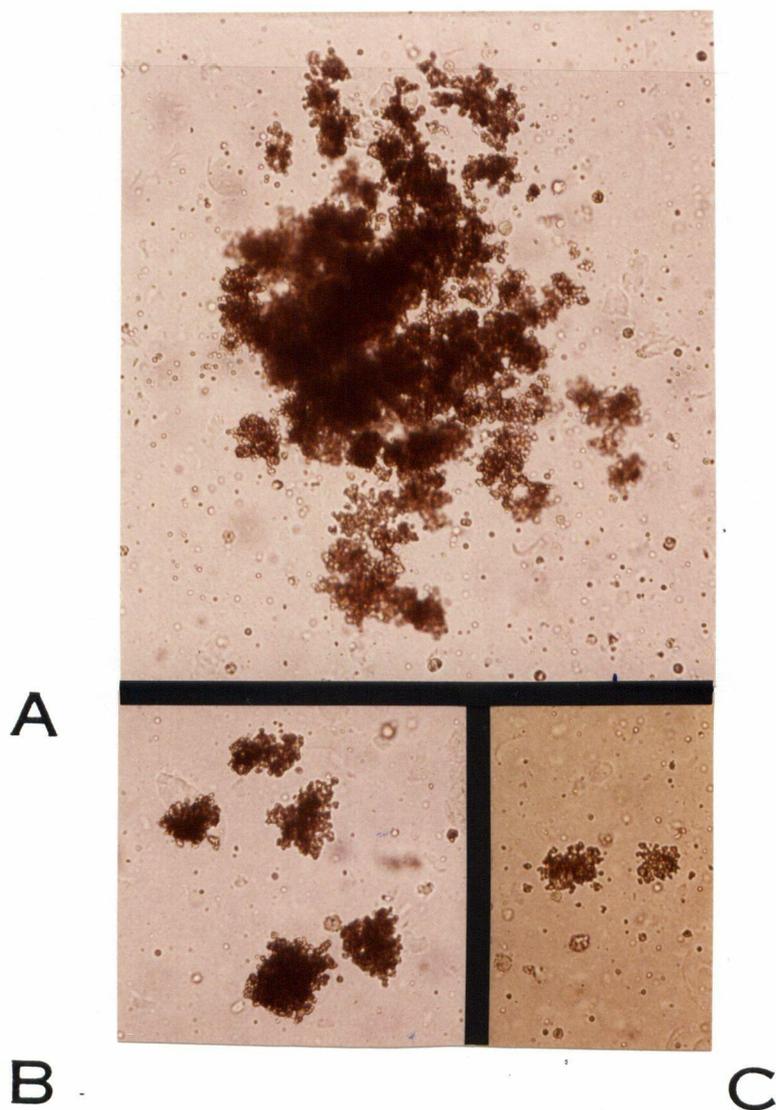


Figure 2. Different sizes of colonies generated in methylcellulose cultures by erythroid progenitors at different stages of differentiation (X80).

A. A large erythroid burst, containing >16 clusters, produced by a primitive BFU-E.

B. A small erythroid burst, consisting of 3-8 clusters, from a mature BFU-E.

C. An erythroid colony, containing 2 clusters, from a CFU-E.

cells were obtained by the addition of pokeweed mitogen stimulated spleen cell conditioned medium (Metcalf et al, 1975). Initially the progeny of the megakaryocytic colony-forming cell (CFU-M) were identified by morphological means, until the development of a plasma clot assay permitted the in situ cytochemical staining of colonies for the presence of acetylcholinesterase (McLeod et al, 1976; Nakeff et al, 1976). Platelet formation from megakaryocytes in colonies has been observed in plasma clot cultures (McLeod et al, 1976).

A plasma clot culture system for the growth of human CFU-M was initially described by Vainchenker and his associates (1979a,b). Subsequently an immunochemical method for the identification of human megakaryocytes was developed, utilizing a highly specific antibody against human platelet glycoproteins (Mazur et al, 1981). More recently larger, compact megakaryocyte colonies have been grown in methylcellulose cultures containing human plasma and PHA-stimulated human leukocyte conditioned media and the cells identified by a positive reaction with antibodies directed against human factor VIII antigen (Messner et al, 1982).

A linear relationship between the number of cells plated and the number of colonies formed is consistent with the clonal origin of each colony (Metcalf et al, 1975; Nakeff et al, 1976). Additional evidence of clonality was obtained from studies utilizing cells from patients with the Tn polyagglutinability syndrome. When examined for the Tn phenotype, megakaryocytes in individual colonies were either Tn⁺ or Tn⁻, providing considerable support for the single cell origin of each colony (Vainchenker et al, 1982).

Multi-lineage colony-forming cells Similar modifications in culture conditions also resulted in the production of mixed colonies containing more

than one lineage of mature cell (Figure 3). Erythroid-megakaryocyte colonies were first described from mouse bone marrow (McLeod et al, 1976).

Subsequently larger mixed colonies, containing cells from all three myeloid cell lines were recognized in cultures of murine fetal liver cells, (Johnson and Metcalf, 1977), and shortly thereafter in cultures of adult mouse bone marrow though at a lower frequency (Hara and Ogawa, 1978; Humphries et al, 1979a; Metcalf et al, 1979). Such colonies were shown to contain CFU-S (Humphries et al, 1979b) and to be derived from cells capable of self-renewal (Humphries et al, 1981). Other mixed colony types, containing different combinations of myeloid lineages have also been described more recently (Nakahata and Ogawa, 1982b; Suda et al, 1983).

Comparable mixed colonies of human origin have been obtained in cultures of human bone marrow, peripheral blood, cord blood and fetal liver. In initial studies a bipotent progenitor was described, capable of giving rise to colonies composed of cells of the granulocytic and erythroid lineage (Fauser and Messner, 1978). The cell of origin was termed a CFU-G/E. Subsequently these authors were able to obtain mixed colonies in human bone marrow cultures in which megakaryocytes and macrophages were present in addition to granulocytes and erythroid cells, and a new term, CFU-GEMM was coined (Fauser and Messner, 1979). In the human system, as in the mouse system, the presence of stimulatory factors present in mitogen stimulated conditioned media was shown to be necessary for the optimal growth of such colonies (Fauser and Messner, 1978; Johnston and Metcalf, 1977).

Comparisons between CFU-S and CFU-GEMM have demonstrated several characteristics in common. These include similarities in proliferative activity of the progenitor compartment in both steady state conditions and perturbed bone marrow, proliferative capacity and some, albeit limited

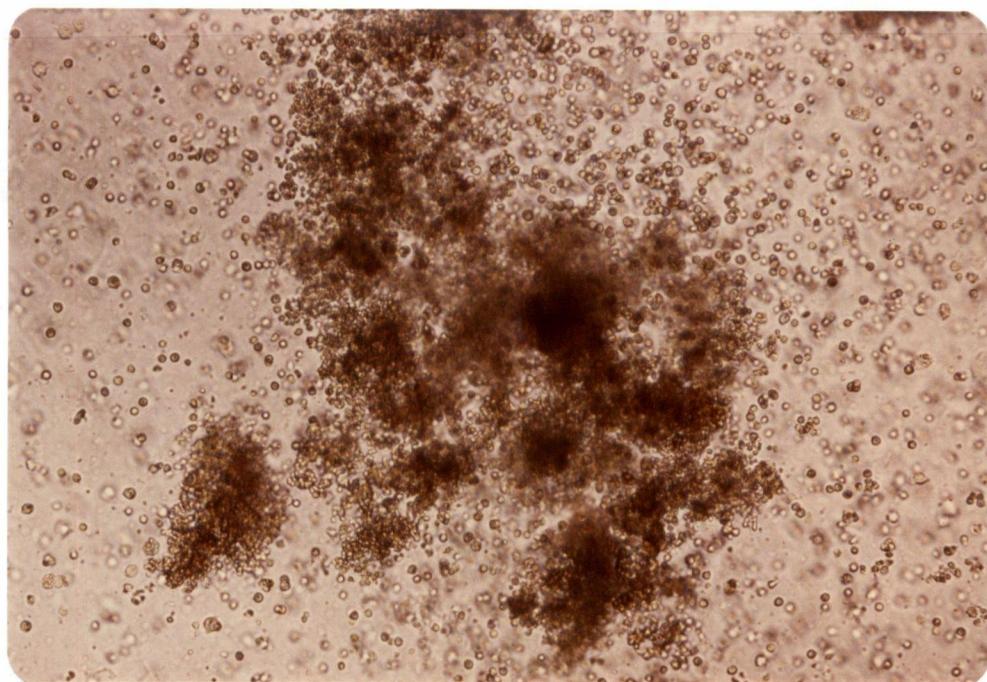


Figure 3. A mixed granulocytic/erythroid colony photographed in a methylcellulose culture after 18 days of incubation. The cell of origin of this colony is termed a CFU-G/E (X120).

ability to self-replicate (Messner and Fauser, 1980; Ash et al, 1981). Recently T lymphocytes (Messner et al, 1981; Fauser and Lohr, 1982; Lim et al, 1984a) and also B cells (Hara, 1983) have been described in a small proportion of multi-lineage myeloid colonies. However, these studies remain controversial and unconfirmed.

Normal blast colony forming cells Another type of colony has recently been described in cultures of normal mouse bone marrow and spleen cells after 16 days of incubation (Nakahata and Ogawa, 1982b). These small colonies, tentatively named stem cell or blast colonies were characterized by the complete absence of terminal differentiation prior to 16 days and their ability, upon replating, to generate large numbers of pure and mixed secondary colonies. These authors suggested that the progenitors of the primary colonies may be located prior to day 9 CFU-S and CFU-GEMM in the hierarchy of stem cell differentiation. Subsequently these investigators demonstrated the presence of progenitors in human umbilical cord blood which gave rise to comparable small undifferentiated colonies after 25 days of incubation (Nakahata and Ogawa, 1982c).

Summary - the hierarchical structure of the hemopoietic system

The myeloid component of normal hemopoiesis as defined by in vivo and in vitro clonal assays of cells produced in vivo is typically subdivided into the following three stages of cellular development, each stage demarcated by major differences in proliferative capacity and differentiation potential (Figure 4). The most primitive compartment consists of pluripotent cells some of which are also capable of generating pluripotent daughter cells. The second compartment consists of progenitors committed to a specific pathway. These cells are the progeny of pluripotent cells in which determination or total lineage restriction has occurred. Committed progenitors may, however,

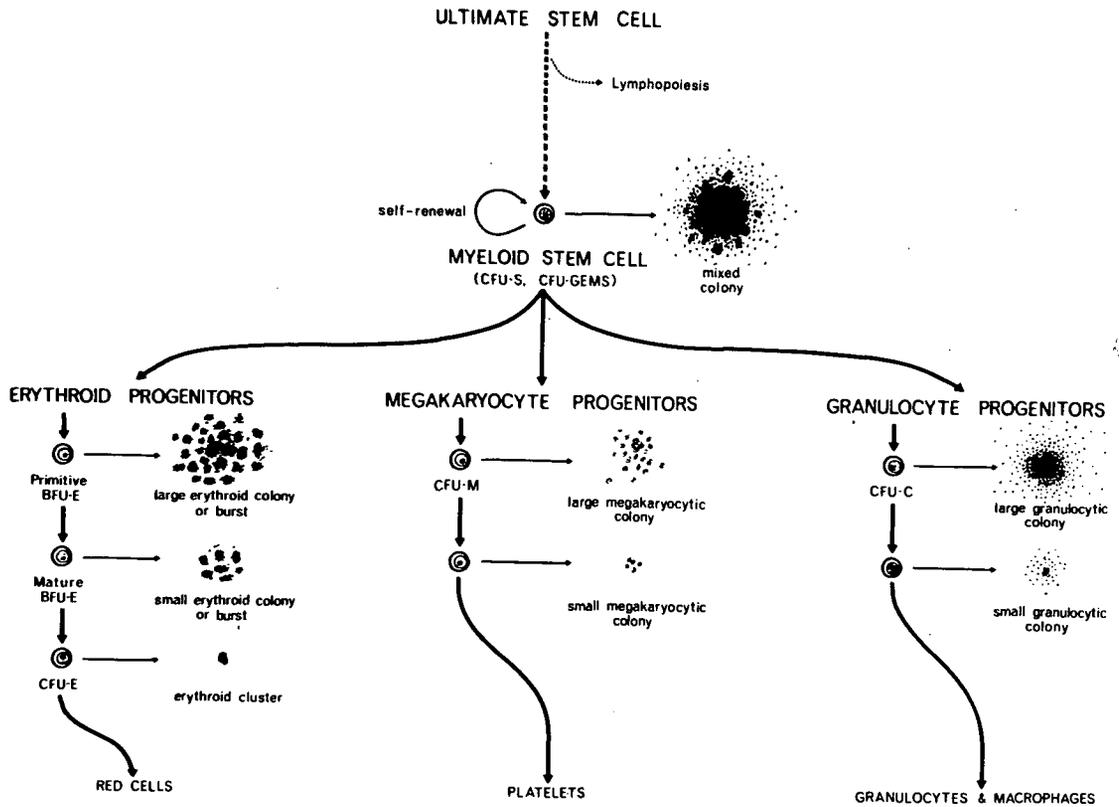


Figure 4. Diagrammatic representation of the hierarchy of hemopoietic progenitor compartments currently identified by colony assay procedures. Colonies are grown in semisolid media, such as methylcellulose or agar, with the addition of serum, essential nutrients and appropriate stimulatory growth factors. According to this model, the state of differentiation of the colony forming cell determines the size of the colony it produces in vitro.

From reference (Eaves and Eaves, 1983); used with permission.

still possess extensive proliferative capacity. The third and largest compartment is composed of cells that are morphologically recognizable. These cells are very limited in their proliferative potential with only a porportion of the compartment undergoing a small number of terminal divisions. Once fully mature these cells are released into the circulation to fulfill their biological role before they die and are replaced by new cells.

The grouping of hemopoietic cells into three compartments is a convenient method of describing the hierarchial structure of the system. However, considerable heterogeneity exists within all compartments and the borders between them may be somewhat blurred when many parameters are examined simultaneously. In addition the existence of bipotent progenitors, such as CFU-G/E or CFU-E/M indicates that intermediate populations of cells may exist between the pluripotent stem cell and the various committed progenitor compartments; i.e. lineage restriction may occur (apparently randomly) in a single or in multiple steps (Nakahata et al, 1982; Suda et al, 1983, 1984 a,b; Lim et al, 1984b; Leary et al, 1984, 1985).

B) Stem Cell Regulation

The nature of the mechanisms controlling cellular proliferation and differentiation is a fundamental problem in biology. In the hemopoietic system our understanding of such regulatory mechanisms affecting the most primitive cells is considerably hampered by our inability to to observe and study the cells of interest directly because they represent rare elements in the entire marrow population and are not physically localized in a fashion analogous to the stem cell populations of other tissues. However, cell cycle studies coupled with cytological examination of the progeny produced by stem cells in vivo suggest three biologically important types of stem cell

decisions that may or may not be independently regulated. These are; 1) decisions affecting changes in proliferative states; 2) decisions to self-renew or differentiate; 3) decisions affecting further restriction of differentiation capacity (Figure 5).

Decisions affecting changes in proliferative status

Lajtha and his associates (1963) were the first to postulate a model in which the majority of the stem cells were envisaged to be in a non-proliferative resting state, termed G_0 , but which could be "triggered" into a cycling state by perturbations in more differentiated cell compartments. Experiments utilizing ^3H -thymidine or other cycle active drugs provided considerable support for this model. Thus, although the majority of the CFU-S in a normal intact mouse are in a resting state, this changes within a few hours after sublethal irradiation (Becker et al, 1965; Eaves and Bruce, 1974), the administration of cycle active cytotoxic drugs (Bruce et al, 1966; Blackett et al, 1968; Vassort et al, 1973; Eaves and Bruce, 1974; Hodgson et al, 1975), endotoxin (Eaves and Bruce, 1972) or bleeding (Duplan and Feinendegen, 1970). Furthermore, while CFU-GEMM in normal humans are predominantly quiescent, the majority are in S-phase during hemopoietic regeneration following marrow transplantation (Fauser and Messner, 1982). These findings indicate that primitive hemopoietic cell populations are capable of changing their proliferative state in response to stimuli associated with terminal cell depletion.

In certain experimental situations, as for example in mice after phenylhydrazine administration (Rencricca et al, 1970; Wright and Lord, 1977) or in experiments using partial body irradiation (Gidali and Lajtha, 1972) the CFU-S in one site may be in active cell cycle, while in another part of the body the CFU-S are quiescent. Such findings may suggest local

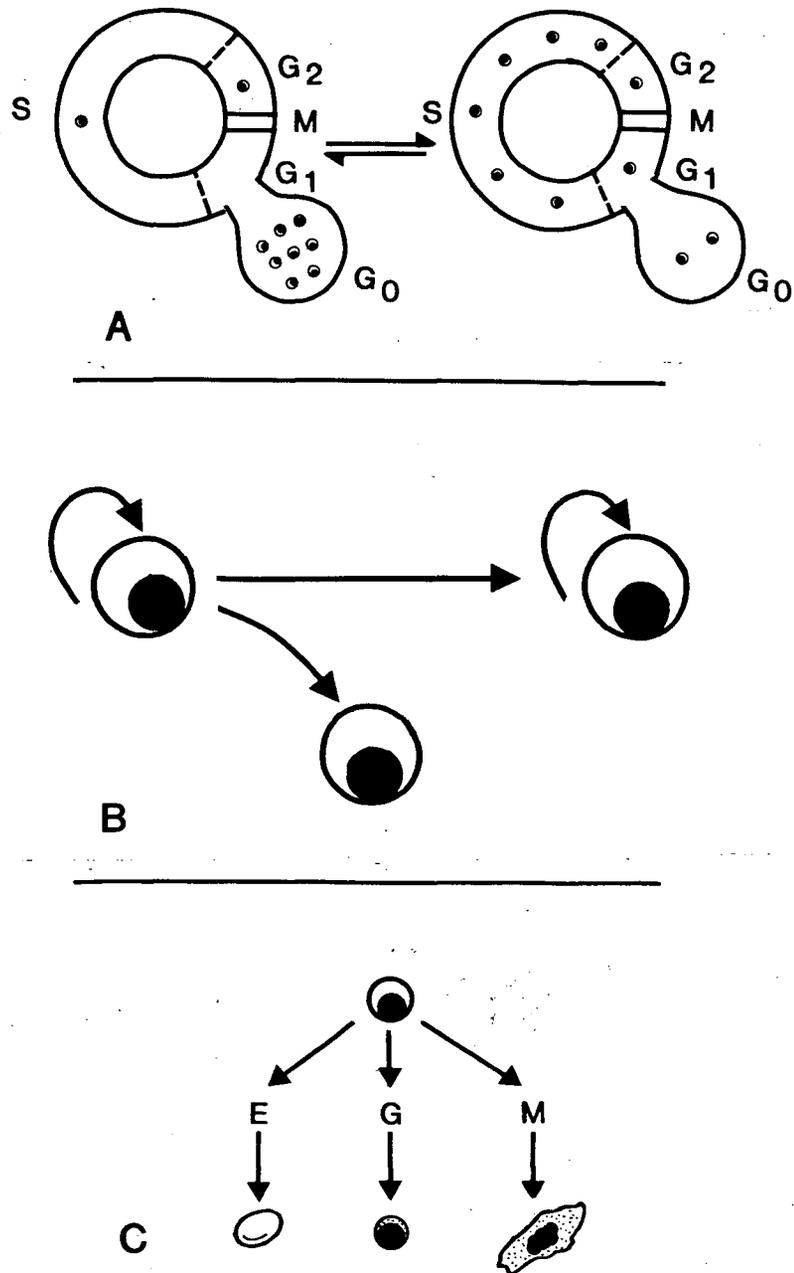


Figure 5. Three types of stem cell transitions where regulatory mechanisms may act to influence stem cell behaviour.

A. Control of stem cell proliferation

B. Self-renewal versus differentiation

C. Restriction of differentiation capacity

regulatory mechanisms in at least some aspects of the control of CFU-S proliferation. However, it has also been shown that depletion of stem cells in one part of the body can result in an increased proliferative activity in an unaffected site, implicating long range humoral control mechanisms (Croizat et al, 1970; Gidali and Lajtha, 1972).

Although the size of the local CFU-S compartment appears to be a common feature associated with stem cell proliferative status, the significance of this association is still not known. A diffusible factor from damaged cells (Frindel et al, 1976) as well as a thymic factor (Lepault et al, 1980) have been suggested as possible long range stimulatory factors. Local negative feedback inhibitors and promoters of cell proliferation have also been described and will be discussed in a later section.

Decisions affecting loss of self-renewal capacity and lineage restriction

By definition commitment involves loss of the capacity to generate new pluripotent daughter cells. Both deterministic and stochastic models have been proposed for the mechanism initiating this restriction process. The deterministic model postulates an inductive effect by external factors on the choice between self-renewal and differentiation. According to this model, originally proposed by Trentin and his associates (1968, 1970) commitment is regulated by the specific inductive microenvironment surrounding each individual stem cell and the spectrum of differentiated daughter cell types produced will thus vary according to local variations in the microenvironment. The ability of individual pluripotent cells to form colonies of different sizes and composition both in vivo (Gregory, 1976) and in vitro (Humphries, 1979a) provide strong evidence against such a model and lent support to the stochastic model proposed first by Till and his associates (1964). According to this model the choice between self-renewal or commitment is made at

random, with a fixed probability for each event (Till et al, 1964; Korn et al, 1973; Till and McCulloch, 1980). This model was originally proposed on the basis of the marked heterogeneity found upon analysis of the cellular content of spleen colonies, particularly in the number of CFU-S contained in individual colonies (Siminovitch et al, 1963). An extension of this model was proposed by Suda and his associates (1983). They suggested that stem cell commitment is a progressive and stochastic process resulting in the gradual restriction of the differentiation potential of the stem cell. This model is based on the demonstration of various combinations of bipotent progenitors which can express colony formation in vitro (Nakahata et al, 1982; Leary et al, 1984; Lim et al, 1984b). Such findings also argue against the stem cell competition model pathway in which specific inducers such as erythropoietin (Ep) and various colony stimulating factors (CSF) were thought to act upon a common stem cell to effect differentiation into a specific pathway (Van Zant and Goldwasser, 1977). This model predicts that competing demands made upon the stem cell resulted in an increase in one cell line at the expense of the other. Although evidence for this was claimed with the demonstration that increasing concentrations of Ep could inhibit granulocytic colony formation (Van Zant and Goldwasser, 1977; 1979), such an effect occurred only when high cell concentrations were plated, suggesting that it was a secondary phenomenon. Other investigators have not observed stem cell competition. Rather, the addition of increasing concentrations of Ep to mixed erythroid colonies resulted in an increase in the mean number of erythroid cells per colony, without affecting other hemopoietic lineages (Metcalf and Johnson, 1979). Other studies have shown that a stimulatory effect on stem cells results in a general increase in all primitive cell types (Gregory and Henkelman, 1977).

However, evidence of some heterogeneity in the self-renewal capacity of different CFU-S and other progenitor cell populations due to non-stochastic processes has also been obtained. Subpopulations of stem cells differing in self-renewal capacity have been demonstrated by experiments using physical separation (Worton et al, 1969), alkylating agents (Schofield and Lajtha, 1973; Morley and Blake, 1974; Botnick et al, 1976; Rosendaal et al, 1979), irradiation (Schofield et al, 1980), serial transplantation (Siminovitch et al, 1964; Micklin and Ogden, 1976; Schofield, 1980), and adherence separation (Mauch et al, 1982 Kerk et al, 1985). Variation in self-renewal capacity have also been found between found between stem cells in the blood and those in the marrow (Micklin and Ogden, 1974). Such findings have led some authors to postulate an age structured stem cell population whose members demonstrate variations in self-renewal capacity based on their proliferative history (Hellman et al, 1978). In addition, environmental factors, such as exogenous CSF concentrations have been shown to modulate the proliferative capacity exhibited by granulocytic/macrophage progenitors (Metcalf, 1985). Whether such mechanisms may also play a role in limiting stem cell behaviour has yet to be determined.

C) Hemopoietic Growth Factors

In a normal individual, regulatory mechanisms operate to maintain the numbers of all circulating mature cell types within narrowly defined limits. Since terminal hemopoietic cells are not capable of division, perturbations which require increased production of mature cells must in some manner affect the status of the more primitive cell compartments. Much of our present knowledge regarding the role of growth factors on the proliferation and survival of hemopoietic cells has been obtained from in vitro clonal assay systems where a requirement for these factors for colony formation was

demonstrated. The heterogenous nature of the various stimulatory factors that could interact with target progenitor cells in the bone marrow would considerably increase the flexibility of the hemopoietic regulatory system, permitting the precise control of mature cell production. However, except for Ep, the in vivo significance of these factors has been highly controversial. More recently, with the availability of pure recombinant growth factors for assessment of in vivo effects (Lee et al, 1985; Rennick et al, 1985; Wong et al, 1985) and the recognition of associations between growth factor independence and the acquisition of a neoplastic phenotype (Cochran et al, 1983; Kelly et al, 1983; Rapp et al, 1985) the significance of most of these factors has assumed a new respectability.

Colony stimulating factors (CSFs)

In the initial description of the in vitro cloning assay for granulocytic progenitors the presence of a cell feeder system was required for colony formation (Bradley and Metcalf, 1966; Pluznick and Sachs, 1966). Subsequently the presence of colony stimulating factor was demonstrated in the medium conditioned by a variety of cells (Bradly and Metcalf, 1966; Austin et al, 1971; Iscove et al, 1971; Parker and Metcalf, 1975; Aye, 1977). More recently purification studies have indicated that the CSFs represent a family of glycoprotein molecules which must be continually present for GM colony growth to proceed (Paran and Sachs, 1968; Stanley et al, 1975; Burgess et al, 1977). The members of the CSF family include 1) M-CSF (or CSF-1) which supports primarily macrophage colony formation (Stanley and Heard, 1977); 2)G-CSF which stimulates almost exclusively granulocytic colony formation in the mouse (Williams et al, 1978a); 3) GM-CSF, originally thought to be specific for granulocyte/macrophage colony progenitors (Burgess et al, 1977); and 4) interleukin-3 (IL-3) or multi-CSF, a factor that stimulates all

types of clonogenic myeloid cells (Metcalf and Johnson, 1978). Besides eliciting responses from different target cell populations, these four CSFs are heterogenous with respect to their molecular weight, carbohydrate content, amino acid sequence and production source (Metcalf, 1985). More recently the genes for human CSF-1, murine and human GM-CSF, murine IL-3 and a human G-CSF like factor have been cloned and the activities of their purified products analyzed both in vivo and in vitro. These and other studies with pure "natural" CSF's have established that none are totally lineage-specific in their effects in vivo and that in both mouse and man GM-CSF and G-CSF can stimulate pluripotent and a variety of lineage restricted progenitor cell types (Gough et al, 1984; Fung et al, 1984; Lee et al, 1985; Hapel et al, 1985; Kawasaki et al, 1985; Rennick et al, 1985; Wong et al, 1985).

Other studies indicate that the CSFs may have similar biochemical effects to other growth factors. For example, the addition of murine CSF-1 to resting target cells results in an increase in DNA synthesis within 10-12 hours (Tushenski and Stanley, 1983), stimulation of the rate of protein synthesis and an inhibition of the rate of intracellular protein degradation. Furthermore GM-CSF also stimulates RNA synthesis and this effect is independent of protein synthesis (Burgess and Metcalf, 1977; Price et al, 1975).

Erythropoietin (Ep) The concept that a humoral factor could regulate red cell production in response to tissue oxygen demands and perturbations in hemostasis was developed early in this century (Carnot and Deflandre, 1906). In the mid 1950s a target cell for this factor was identified by studies in polycythemic mice (Jacobson et al, 1957) and termed the Ep-responsive cell. The demonstration that maturing erythroblasts disappear rapidly from the

marrow and spleen when Ep is removed, but reappear within 48 hours after Ep administration identified the ERC as a fairly mature progenitor cell of the erythroid series.

Ep has been purified to homogeneity and studies on its biochemistry have characterized the molecule as an acidic glycoprotein with a molecular weight of about 34,000 daltons (Jacobs et al, 1985). The gene has recently been cloned and the expressed product shown to have the same activity in vivo and in vitro initially ascribed to the naturally produced material. Ep is normally made by the kidney and during fetal life is also produced in the liver.

When in vitro colony assays for erythroid progenitors were developed a critical role for Ep in the regulation of erythropoiesis was reaffirmed by the strict dependency of CFU-E colony formation on the presence of Ep in the culture (Stephanson, 1971). Reduction of Ep levels in mice resulted in a substantial decrease in the number of CFU-E in both spleen and marrow, and this effect could be reversed by Ep administration (Gregory et al, 1973; Axelrad et al, 1974). Ep dose response studies have demonstrated that CFU-E are the most sensitive erythroid progenitor class and can respond to very low levels of the hormone (Gregory et al, 1973).

Other studies have indicated that the immediate precursors of CFU-E, the mature BFU-E may also be regulated in part by Ep. Mature BFU-E colony formation is also dependent on the presence of Ep, although a considerably higher concentration of the hormone is required for optimal cloning efficiency in comparison to CFU-E (Gregory et al, 1976).

The molecular mechanisms of Ep induced red cell differentiation are not well known. Since the cycling activity of residual CFU-E is not altered in polycythemic mice (Iscove, 1977) it has been suggested that Ep acts to

promote the survival of CFU-E thus permitting the completion of their predetermined differentiation program (Eaves et al, 1979a). Other studies using radioactive cloned globin gene fragments have shown that Ep may directly induce globin gene transcription (Bondurant et al, 1985). A mitogenic effect of Ep at the level of mature BFU-E has been suggested by the demonstration that some of these cells can respond to erythropoietic stimulation by increasing their cell cycle activity (Adamson et al, 1978).

Other factors A number of inhibitory factors have also been implicated in the regulation of granulopoiesis. Colony inhibitory activity (CIA) derived from polymorphonuclear neutrophils is a specific inhibitor of CSF production by monocytes in vitro (Bruch et al, 1978; Broxmeyer et al, 1977a). Subsequently this glycoprotein was identified as lactoferrin (Broxmeyer et al, 1978). In addition to its suppressive effects on the release of CSF, lactoferrin may inhibit monocytic and macrophage production of acidic isoferritins (Broxmeyer et al, 1984a). Lactoferrin is present in the plasma at a much higher concentration than that required to inhibit CSF production in vitro, making its in vivo significance unclear (Bennett et al, 1978). However, in vivo administration of purified, endotoxin-free lactoferrin was shown to decrease the number of multipotential, erythroid and granulocytic precursors in murine marrow and spleen and to arrest the turnover of these normally cycling progenitor cells (Broxmeyer et al, 1984b; Lu et al, 1983).

Prostaglandins of the E series appear to have an inhibitory effect on CFU-C number in vitro (Pelus et al, 1979; Williams, 1979). Macrophages elaborate PGE in response to CSF stimulation in vitro, suggesting an interplay between CSF and PGE may have a role in granulocyte regulation (Kurland et al, 1978). Other inhibitors of CFU-C colony formation include the adenosine nucleotides (Kurland et al, 1977; Taettle and Mendelson, 1980),

interferon (McNeil and Fleming, 1971, Greenberg and Mosney, 1977; Kimpel et al, 1982;) and lipoproteins (Douay et al, 1983) but in these cases no experimental assessment of their role in normal granulopoiesis has been reported.

Although lactoferrin has no effect on erythropoiesis, an inhibitory effect of acidic isoferritin on BFU-E proliferation has been demonstrated (Lu et al, 1983). In contrast to its effect on granulopoiesis, PGE stimulates the growth and differentiation of BFU-E (Chan et al, 1980; Rossi et al, 1980; Degowin and Gibson, 1981). PDGF has also been shown to increase the proliferation of erythropoietic cells in vitro (Dainiak et al, 1983).

Wright and his associates have described two factors that were capable of specifically inhibiting or stimulating the proliferative activity of CFU-S (Wright and Lord, 1977; 1978). The stimulatory activity was extracted from regenerating murine bone marrow where a high proportion of CFU-S were in S phase (Lord et al, 1977a), while the inhibitory substance was obtained from normal bone marrow when the majority of CFU-S were quiescent (Lord et al, 1976). Subsequently these investigators demonstrated the presence of both factors in numerous other hemopoietic tissues of human and murine origin. In each instance the relative proportion of each factor was correlated with the proliferative status of the CFU-S in the tissue (Wright and Lord, 1979). In addition, each factor could be used in competition with the other to reversibly alter the proliferative activity of the stem cell in either a positive or negative fashion (Lord et al, 1977b). These authors suggested that the relative concentration of the two factors in vivo could regulate the level of stem cell proliferation (Lord and Wright, 1982).

Extensive work in Byron's laboratory has demonstrated that β -adrenergic agents (Byron, 1972, Byron, 1975), cholinergic agents (Byron, 1975) and

histamine (Byron, 1977a) could increase the proportion of stem cells in S-phase in vitro, and their effects appear to be mediated through the cyclase system. Since the addition of cyclic nucleotides will also increase CFU-S cycling (Byron, 1971) a regulatory role for phosphodiesterase in maintaining stem cells in a quiescent state was postulated. Androgens (Byron, 1970, 1971, 1972), prostaglandin E₂ (Feher and Gidali, 1974; Lu et al, 1984. and PDGF (Michalewicz et al, 1985) have also demonstrated a stimulatory effect on stem cell cycling. In contrast, interferon appears to decrease the clonogenic efficiency of CFU-GEMM (Neumann and Fauser, 1982).

Role of the stroma The main site for hemopoiesis in the adult is the bone marrow where numerous cell types such as endothelial, adventitial reticular cells, adipocytes, fibroblasts and macrophages form a complex three-dimensional matrix. Morphological studies have shown that hemopoietic cells in various stages of differentiation and maturation form specific interactions with various of these components of the marrow stroma. For example, areas of active erythropoiesis are found in close proximity to the sinusoidal endothelium, while granulocytic cells are observed in association with the extravascular reticulum cells (Sorrell and Weiss, 1980). In rodent marrow erythroid progenitors have been associated with a macrophage-like acid phosphatase positive cell (Westin and Bainton, 1979; Ben-Ishay and Yoffey, 1972) and granulocyte precursors with an alkaline phosphatase positive reticulum cell (Westin and Bainton, 1979). In addition, adipocytes are often found in areas of active granulopoiesis (Weiss, 1980).

Several experimental observations provided indirect evidence for a regulatory role of the bone marrow stroma in vivo. For example, hemopoietic stem cells circulate freely (Everett and Perkins, 1979) but only proliferate in certain organs such as the bone marrow and spleen (Till and McCulloch,

1961). Hemopoiesis in bone marrow that has been irradiated or mechanically damaged only resumes after a functional stromal architecture is re-established (Tavassoli and Crosby, 1968). A similar situation is seen in the regenerating marrow of leukemic patients after marrow transplantation or following extensive chemotherapy (Cline et al, 1977). In these patients hemopoiesis resumes as discrete foci of regenerating hemopoietic precursors in specific micro-anatomical relationships with stromal elements comparable to those observed during fetal development (Islam et al, 1980, 1984).

Similar observations led Trentin and his co-workers to propose the concept of a hemopoietic inductive microenvironment (HIM) which could induce the differentiation of pluripotent stem cells along specific committed cell lines (Trentin, 1970, 1971). They found that in irradiated recipients of hemopoietic cell suspensions colony formation in the spleen was predominantly erythropoietic, while granulocytic colonies were more common in the bone marrow. Similar erythropoietic/granulocytic ratios were also found in secondary recipients whether the injected cell suspension was obtained from an erythroid or a granulocytic colony. According to this concept mixed colonies arose when a colony enlarged and encountered new areas of stroma with different microenvironmental influences (Curry and Trentin, 1967). Indeed, direct visualization of the inductive effect of stroma on the morphology of spleen colonies was seen in experiments where plugs of bone marrow stroma were implanted within the spleen. In such spleens, when individual colonies were located at the junction of marrow and spleen stroma, that portion of the colony within the spleen stroma was predominantly erythroid, while the portion of the same colony in association with marrow stroma was predominantly granulocytic (Wolf and Trentin, 1968). However, in studies where the contents of individual spleen colonies were replated in

vitro the heterogenous nature of the secondary colonies produced suggest that these apparent effects of the stroma on stem cell differentiation may be explained by selective amplification of later cell types (Gregory, 1973: Gregory et al, 1974).

An extrinsic role of the microenvironment on hemopoiesis has also been suggested by studies on the genetically anemic Sl/Sl^d mouse. Stem cells from Sl/Sl^d mice are capable of forming spleen colonies when injected into +/- normal syngeneic mice, but transplanted stem cells from +/- littermates do not form spleen colonies in Sl/Sl^d mice (McCulloch et al, 1965), suggesting that the microenvironment in Sl/Sl^d mice may be responsible for the proliferative failure of the transplanted normal stem cell growth. The macrocytic anemia present in these mice can be cured by transplant of normal spleen tissue, but not by infusions of normal marrow cells. The normal implanted spleen tissue displays areas of active hemopoiesis in contrast to the adjoining Sl/Sl^d stroma which remains in a quiescent state (Wolf, 1978).

2) THE CELL CYCLE

Much of our knowledge of the cell cycle and growth kinetics of homogeneous cell populations was initially obtained from labelling studies utilizing tritiated thymidine, particularly in conjunction with the technique of autoradiography. More recently, flow cytometry has enabled more rapid and precise measurements of cell cycle parameters for homogeneous cell suspensions or suspensions in which the cell population of interest can be identified in the flow cytometer by some other unique property. In the case of hemopoietic cell suspensions where the cells of interest cannot yet be identified by such means, inhibition of colony formation has been used to distinguish S-phase cells exposed to lethal S-phase specific agents such as high specific activity tritiated thymidine or various drugs.

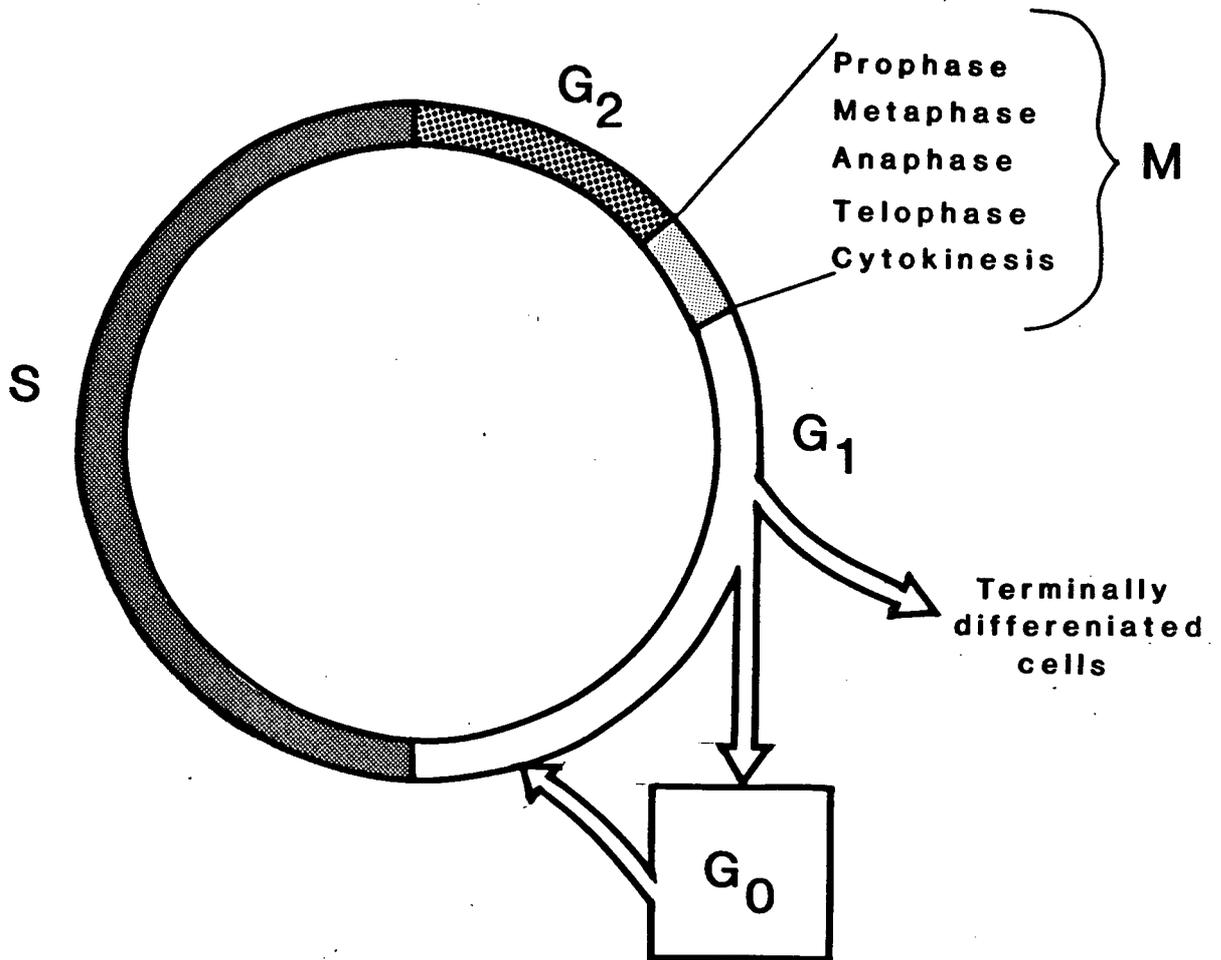


Figure 6. The cell cycle. Terminally differentiated cells are incapable of further division while cells in G₀ may be quiescent for varying periods of time before responding to an appropriate stimulus to enter the cell cycle and divide again.

A) Description and Regulation of the Cell Cycle

The cell cycle concept originated in the early 1950's when it was found that the cell made new DNA at a discrete time between divisions, subsequently referred to as "S" phase (Howard and Pelc, 1953). After S there is a "gap" of varying length G_2 , during which the cell has a $4n$ DNA content, before proceeding to the next phase, termed M (for mitosis) or D (for division). Following cell division, the cell enters the G_1 phase. This part of the cell cycle may vary extensively in length, not only between different cell types, but between members of the same cell population. Certain cells, such as blastomere cells, do not enter a G_1 phase (G_1^- cells) but proceed directly into S phase after division (Prescott, 1982). In most cells however, the four phases G_1, S, G_2 and M constitute the cell cycle (see review by Wheatly, 1982). Despite the popular nomenclature however, it must be kept in mind that the processes of growth, synthesis and division are not truly cyclical, as considerable variability may occur in successive divisions.

G_1 The cell enters G_1 as soon as division is completed. Some of the early events in G_1 include the synthesis of membrane components, such as phospholipids, glycoproteins, and sialic acid. As G_1 proceeds, the cell may develop many finger like microvilli, which can be retracted at division to provide the extra surface covering capacity required when a spherical object divides into two approximately equal parts.

Most G_1 cells undergo steady linear growth until the time at which S phase is initiated. Certain enzymes necessary for the process of DNA synthesis, such as thymidine kinase, thymidylate synthetase and others increase sharply in cells during the last part of G_1 . Inhibitors of protein or RNA synthesis will block the formation of these enzymes and prevent the cells from entering S. Once a cell has synthesized all the basic

requirements for DNA replication it has passed the point of commitment, but the nature of the trigger that permits progression into the next phase is unknown.

S The mammalian cell contains approximately 2000 initiation sites where the two strands of parental DNA are separated to permit template formation. The sequence of initiation from site to site follows a rigid pattern that is repeated with remarkable consistency cycle after cycle. The rate of DNA synthesis accelerates at the beginning of S-phase, reaches a maximum and then decreases. Once initiated DNA synthesis normally proceeds until completion.

During S-phase the cell continues to grow and synthesis of subcellular organelles occurs, along with changes in surface morphology. In addition replication of DNA requires synthesis of histones, highly basic proteins rich in lysine and arginine which are characteristically associated with DNA.

G₂ G₂, the period from S phase to division is poorly understood. Both protein and RNA synthesis are necessary for an interval after the start of G₂. Soon after, RNA synthesis is no longer needed, and then closer to mitosis, protein synthesis ceases.

Once the cell has progressed into G₂, it will reach division within a fairly well proscribed period of time. A certain minimum span of time is required for most cell types however, suggesting that certain definite preparations must be undertaken before mitosis can begin. Current opinion suggests the concurrence of a number of discrete processes, such as absolute size of the cell, right concentration of important molecules, and the assembly of macromolecules into specific structures are necessary before division begins. A second possibility is that the cell requires a certain amount of time between S-phase and division to edit the newly synthesized DNA and to remove any errors in copying.

Mitosis Cell division in eukaryotes is mechanically a very complex process. First, the chromosomes must line up, separate, and then move to the opposite sides of the cell. Then, during the process of cytokinesis the cell must divide in such a manner as to ensure an equal distribution of all necessary cytoplasmic constituents and organelles. The cellular machinery responsible for this complex task is the mitotic apparatus.

The process of mitosis is divided into five stages; prophase, metaphase, anaphase, telophase and cytokinesis. During prophase the diffuse chromatin of interphase condenses into well defined chromosomes, each consisting of two sister chromatids joined by a centromere. The nucleolus, nuclear membrane and cytoskeleton disassemble, and construction of the mitotic apparatus begins. The centrioles, replicated during S phase now separate, with each pair and its radial array of microtubules moving apart to form a bipolar mitotic spindle. As metaphase is reached the chromosomes are aligned at the metaphase plate by the interaction of their kinetochore fibers with the mitotic spindle. Anaphase begins when the chromosomes begin to move apart, and lasts only for a few minutes. As the chromosomes move apart, the kinetochore fibers shorten, the spindle fibers elongate, and the two poles of the polar spindle move further apart. The sudden and dramatic movement of chromosomes appears to require little energy and may be due to disassembly of the microtubules at the polar end of the mitotic spindle. During telophase many of the events occurring in prophase are reversed; the kinetochore fibers disappear, the spindle disperses, a new nuclear envelope forms around each group of daughter chromatids, the condensed chromatids expand, and nucleoli begin to reassemble. The process of cleavage begins in late anaphase when a contractile ring forms in the region of the cell periphery around the area of the metaphase plate. This contractile ring consists of a thickened area of

filamentous cytoplasm, containing fibronectin and actin, just beneath the surface of the cell membrane. As it contracts, a cleavage furrow is formed, which continues until only the pole to pole fibers of the mitotic spindle are contained within the ring, forming the midbody. Eventually the bridge breaks apart, and two new separate daughter cells are formed.

The G_0 state Some normal cells may make a choice between proliferation or quiescence when they are in the G_1 phase of the cell cycle. According to one view (Lajtha, 1963), quiescent cells withdraw from the cell cycle to enter a qualitatively distinct G_0 state. Definite biochemical differences do exist between G_0 and G_1 cells, such as polyribosome content (Becker et al, 1973), chromatin template activity (Rovera and Baserga, 1973), and membrane transport (Saunders and Pardee, 1972). Cell fusion studies suggest that quiescent cells may be in a distinct arrested state because they contain an inhibitor of DNA synthesis not present in cycling cells (Yanishivsky and Stein, 1980). Cells emerging from the G_0 state are more sensitive to certain drugs than are actively cycling cells (Yen et al, 1978) and require a longer lag time to initiate S-phase suggesting that specific biochemical events are required to leave the G_0 state. Cells may enter G_0 under a variety of adverse conditions, including serum limitation, nutrient deprivation, high cell density, and certain drugs. In the intact animal quiescent cells may serve as a reserve population which can enter the proliferating pool when required, as for example, hemopoietic stem cells.

B) Factors Controlling Cell Proliferation

The different cell populations in most multicellular animals show different rates of turnover. Some cell types, such as neurons, skeletal muscle cells and red blood cells cannot divide at all. Other cell populations such as the epithelial stem cells of the skin and the gut appear

to consist almost entirely of cells that divide rapidly and continuously during the entire lifespan of the individual. Most cell populations however, fall between these extremes. In every tissue a balance must exist between the rate of cell turnover and the rate of cell loss, not only cell loss due to differentiation but also cell loss due to natural or injury related death processes. Various factors have been implicated in maintaining this balance. These factors may induce quiescence, result in withdrawal from the G_0 state, play a role in regulation of G_1 -S transition, or simply serve to maintain cell viability throughout the cell cycle

Density dependent inhibition Normal anchorage-dependent cells in culture will cease mitotic activity at confluence. The final cell density is a function of the concentration of mitogens in the growth medium (Scher et al, 1979) or the serum concentration (Holley, 1975). A number of mechanisms have been attributed to this phenomenon of density dependent inhibition, including cell-cell contact (Bunge et al, 1979; Lieberman and Glaser, 1981), changes in cell shape (Fox et al, 1979), the accumulation of inhibitors (Holley, et al, 1978) and depletion by the cells of media components, such as necessary nutrients or growth regulatory factors, or their restriction to the cell as a consequence of crowding (Dulbecco and Stoker, 1970; Stoker and Piggott, 1974).

Recent studies have focussed on the role of membranes in producing density dependent inhibition. Addition of partially purified membrane fractions from confluent 3T3 cells to growing 3T3 cells resulted in G_0 arrest of 50% of the cycling cells but did not inhibit growth of SV40 transformed 3T3 cells (Whittenberger et al, 1977). In contrast, no inhibition of DNA synthesis was seen when the surface membrane fraction from proliferating 3T3 were used. Further studies indicated that the inhibitory component was a

heat labile, nondialyzable fraction of the membrane (Whittenberger et al, 1978, 1979). Growth inhibitors on the cell surface have been described by several other groups (Yeh and Fisher, 1969; Natraj and Daltra, 1978; Lieberman et al, 1981). Data from these experiments are compatible with the hypothesis that receptor molecules on the cell surface mediate cell-to-cell contact, and these receptor molecules may be released into the media.

Protein metabolism Studies utilizing inhibitors of protein synthesis in cultured mammalian cells have shown that continuing protein synthesis is necessary to maintain progression through the cell cycle. A decrease in the rate of protein accumulation results in extension of the G_1 period (Brooks, 1977; Baxter and Stamma, 1978). Conversely, quiescent cells show little or no net accumulation of protein (Castor, 1977; Stainers et al, 1977a), but will increase protein synthesis when stimulated to regrow. In certain mammalian cells that don't have a G_1 , treatment with inhibitors of protein synthesis delays entry into S-phase, thereby creating a G_1 interval (Liskay et al, 1980). These data would support the hypothesis that mammalian cells must satisfy a growth/protein related requirement for entry into S-phase.

Cyclic nucleotides The role of cyclic nucleotides in the regulation of cell growth has been studied extensively in recent years. An early hypothesis associated cAMP and cGMP as antagonists controlling cell proliferation where cAMP acted to arrest cell growth, whereas cGMP stimulated growth (Goldberg et al, 1974). However, although cyclic nucleotides may have an important role in regulating other cellular processes, more recent work does not support this hypothesis (Zeilig and Goldberg, 1977). Nevertheless the level of cAMP does fluctuate depending on the growth state of the cell (Pastan et al, 1975) and the stages of the cell cycle (Burger et al, 1972; Costa et al, 1976) indicating that cAMP may regulate specific stages in the

growth cycle. The role of cGMP in cell growth regulation however, remains unclear.

Polyamines and ornithine decarboxylase The polyamines putrescine, spermine and spermidine are involved in several aspects of nucleic acid and protein metabolism. Increased levels of polyamines and of ornithine decarboxylase, the key enzyme regulating their synthesis, occur very soon after stimulation of cells (Pardee et al, 1978; Rothstein, 1982). In some cells increased levels of spermine and spermidine are correlated with passage through G₁ and entry into S and may be involved in DNA synthesis (Heby et al, 1975; Boyton et al, 1976) Prior addition of inhibitors of ornithine decarboxylase to quiescent cultures prevents the entry of cells into S-phase induced by serum stimulation (Boyton et al, 1976). Conversely, agents that stimulate cell growth, such as hormones (Nissley et al, 1976), tumor promoting chemicals (Yuspa et al, 1976), and tumor viruses (Gazdar et al, 1976) increase ornithine decarboxylase activity. However, although numerous experiments have shown that in some cells a certain minimum level of polyamines is necessary for progression through the cell cycle, increased levels of polyamines and heightened ornithine decarboxylase activity are not always associated with enhanced mitotic activity (Niskanen et al, 1983). Therefore any true regulatory role for polyamines in cell growth remains unclear.

Ions Calcium, and its ubiquitous binding protein calmodulin have been implicated in the regulation or modulation of various stages in cell proliferation including division (Welsh et al, 1979) and initiation of DNA synthesis (MacManus et al, 1978). Calmodulin and calcium may act by regulating in certain cells, the fluxes of other ions like K⁺, which in turn affect glycolysis, and protein and nucleic acid synthesis (Durham, 1978).

Many mitogens such as serum and growth factors may act to mobilize cations in cells (Ralph, 1983). In calcium free media, serum refeeding has no effect, but stimulation will occur when calcium is added (Lobue and Lobue, 1984). Increases in the intracellular levels of calcium could regulate the second messenger function of cAMP, either by inhibiting adenylate cyclase, or stimulating cyclic nucleotide phosphodiesterase (Ross and Gilman, 1980).

An increase in DNA synthesis also occurs when zinc, cadmium and mercury are added to cultured cells (Rubin, 1975a) while magnesium deprivation induces quiescence in rapidly dividing chick embryo fibroblasts (Rubin, 1975b). A regulatory role for magnesium has been proposed in those metabolic pathways where the rate limiting reactions are transphosphorylation ones.

Growth factors and hormones Since the early days of cell culture serum has been a necessary component of culture systems. Attempts to define factors in serum that induce DNA synthesis in fibroblasts has led to the discovery of a number of specific polypeptide growth factors. These include insulin-like growth factors I and II, somatomedins C and A, epidermal growth factor (EGF) and platelet derived growth factor (PDGF) (see review by Antoniades and Owen, 1982). These factors initiate their effects by binding to high affinity membrane receptors. This event is closely followed by ion movements associated with changes in membrane permeability. The steps leading from these preliminary events to the initiation of DNA synthesis and mitosis are obscure, although in the case of EGF and PDGF the activation of protein kinase, resulting in the phosphorylation of tyrosine residues of membrane proteins appears to be a critical event. Other events observed within minutes after binding of the factor include phospholipase A₂ activation, effects on cyclic nucleotide concentrations, and increased uptake of amino acids, phosphate and glucose. Growth factors activate the machinery

for protein synthesis, and transcription, RNA processing, polysome assembly and the formation of polypeptide chains are also stimulated after treatment with these factors (Antoniades and Owen, 1982).

The prereplicative phase (G_0/G_1) of the cell cycle has been suggested as the primary site of growth factor action (Pardee, 1978). Proliferation is initiated by "competence" factors which permit quiescent cells to enter the cell cycle and to respond to "progression factors" which enable the competent cell to progress toward the S-phase of the cell cycle. In the absence of competence factors cells are unable to proliferate in response to progression factors (Pledger et al, 1977). Competence and progression are two functionally distinct subphases of G_0/G_1 . Growth factors such as PDGF, fibroblast growth factor, and macrophage-derived growth factor do not function well as progression factors. (Stiles et al, 1979, Wharton et al, 1982). Competence is transferable from one cell to another by fusion suggesting that the competent state may require the activation or synthesis of a cytoplasmic factor (Smith and Stiles, 1981). In contrast, progression factors like EGF, must be present continuously until S-phase is reached (Lembach, 1976).

The recent demonstration that the amino acid sequence of PDGF shows considerable homology to that of the transforming protein (p28^{sis}) of Simian sarcoma virus (Waterfield et al, 1983) implies that growth factors play an important role in alterations in cell proliferation behaviour. In some cell lines viral transformation may abrogate the requirement for competency perhaps by directly activating genes required for the induction of the competent state. For example, PDGF has been shown to be capable of inducing the competence genes c-myc, the cellular homologue of the avian transforming oncogene v-myc (Cochran et al, 1983; Kelly et al, 1983) and c-fos, the

cellular homologue of v-fos, the transforming gene of the FBJ sarcoma virus (Cochran et al, 1984). In addition, infection of hemopoietic cells by recombinant murine retrovirus expressing v-myc oncogenes has been shown to result in the abrogation of the requirement for IL-3 (Rapp et al, 1985).

C) Models of Cell Cycle Regulation

In most cell culture systems the M, S and G₂ phases of the cell cycle are fairly consistent in length. The G₁ phase however, is quite variable even between individual cells in the same population. Adverse culture conditions can greatly extend the G₁ period. Most models dealing with the control of cell proliferation therefore propose G₁ as the most likely part of the cell cycle where growth factors act.

Transition probability model According to this model the cell cycle is divided into two parts. A cell at birth is in the A state, where its activity is not directed towards replication (Smith and Martin, 1973, 1974). The cell remains in the A state until a transition occurs upon which it enters B state, and proceeds to mitosis through the usual sequence of stages G₁, S, G₂, and M. Once initiated the cell in the B state progresses towards completion within a defined time T_B. The transition from A to B state occurs at random so that the probability of the transition occurring in unit time is constant. Environmental factors influence proliferation rate by altering the transition probability K_A (Shields and Smith, 1977). Quiescent cells are described by this model as cells in the A state which have a very low K_A. As originally proposed, this model takes as implicit the constancy of the B phase, although other investigators have demonstrated that variability in T_B can account for a significant part of the total variability in the intermitotic time (Shields and Smith, 1977). In addition the original transition model could not account for the long lag period, on average ten

hours, that occurs between stimulation of the cells and the initiation of DNA synthesis. To accommodate these findings, Brooks et al (1980) postulated the two transition model. In this version quiescent cells are located in an indeterminate state Q, and pass from this state to A by completing a lengthy process L, of duration T_L , which occupies most of the lag time. The process L is initiated at random with a rate constant K_Q and is the first random transition. Once L is completed the cells enter the A state, which as in the previous model they leave at random with a rate constant K_A to enter the B state. Both K_Q and K_A depend on the environmental conditions. The initiation of centriole replication and the separation of the mother and daughter centers have been proposed as potential candidates for the two random transitions (Brooks, 1981).

Restriction point model From the kinetics with which quiescent cells enter S-phase after the reversal of adverse environmental conditions, Pardee (1974) proposed that a single major regulatory event must occur in G_1 if a cell is to progress to S-phase. This restriction or R point corresponds to the first of a series of biochemical events, stimulated by environmental factors which leads to the initiation of DNA synthesis. According to this model, a cell will pass through the R point if conditions are favourable. Once the R point is past, the cell is committed to complete the rest of the cycle. Under adverse conditions a cell cannot pass through R, and must then cease proliferation, remaining with a G_1 DNA content. In this model only one control point exists and it is responsive to many environmental factors.

A recent addition to the model suggests that the R point may consist of the synthesis of a labile regulatory protein to a critical threshold necessary for commitment to reproduction (Rossow et al, 1979). A possible candidate for this R protein is a cytoplasmic inducer present in S-phase

cells which can stimulate nuclei from non-S cells to synthesize DNA (Roa and Johnson, 1970; Yanishevsky and Prescott, 1978). More recently, the demonstration that the 53 kilodalton T protein originally found in SV40 transformed cells is also present in high amounts in rapidly proliferating normal human cells (Dippold et al, 1981) has led to the hypothesis that it may be the R protein (Campiri et al, 1982).

D) Principles of the ^3H -Thymidine Cell Suicide Assay

Since thymidine is a specific precursor of DNA, damage to the DNA is the cause of the detrimental effects of tritiated thymidine incorporation, which can result in cell killing, mutation, and loss of reproductive capability. Incorporation of radionuclides exposes DNA to radiation and/or transmutation effects, and therefore interferes with DNA replication, and affects to DNA structure in a manner that alters genetic function.

The energy from incorporated tritium (^3H), like that of other beta emitters is absorbed over a defined, relatively short path from the decay event. The radiation effects are thus produced in close proximity to this path. In the case of ^3H -thymidine, the radiation is limited to the site of radionuclide incorporation within the cell, i.e. the nucleus. The number of cells at risk in a cell suspension from exposure to solutions containing high specific activity ^3H -thymidine will therefore be determined by the number of cells synthesizing nucleic acids at the time the labeled precursor is present (see NCRP report, 1969).

Characteristics of tritium and thymidine

^3H ^3H has a half-life of 12.26 years and decays to ^3He by emitting beta particles with a mean energy of 5.7 kev and a maximum energy of 18.6 kev. In water the mean range of the beta particle is .69 μm . ^3H has been used to trace label a wide variety of organic molecules by exchange reactions in which some of the hydrogen atoms in the molecules are replaced by ^3H .

Thymidine In the adult organism about 20g of DNA is synthesized in one day, mostly in the GI tract, skin, and bone marrow, resulting in a daily requirement of about 4g of thymidine. This amount is provided by the body by de novo synthesis from deoxyuridylic acid and by reutilization of thymidine from dead cells (Figure 7).

Thymidine may be labelled with ^3H at carbon 6, or in the methyl group attached to carbon 5. When cells in S-phase are exposed to ^3H -thymidine, either in vivo by injection, or directly in vitro, the labeled nucleoside easily crosses the membrane in an energy requiring process controlled by thymidine kinase (Cleaver, 1967).

B) Modes of radiation injury

The decay of ^3H incorporated into DNA produces the following two main types of effects:

Transmutation When ^3H decays it is converted to helium, a process referred to as transmutation. The newly formed nuclei require a set of balance electrons different from those in the old nuclei. Transmutation will therefore cause a sudden disturbance in the orbital electrons followed by a rearrangement to form a stable configuration. This rearrangement may then inflict damage to the molecule containing the decayed atoms (Carsten, 1979). Transmutation effects are localized to the immediate site of nuclear decay, and are limited to those molecules to which the radionuclide is attached.

Since the daughter nuclide of ^3H is a noble gas, it will not form a stable chemical bond. Hence transmutation of ^3H attached to a carbon results in the loss of the daughter nuclide and production of a reactive carbonium ion. The effects of ^3H decay have been studied in cultured Chinese hamster cells (Cleaver, 1977) and it was shown that transmutation of ^3H to ^3He in the carbon 6 position in thymine in the DNA resulted in single-strand and double-

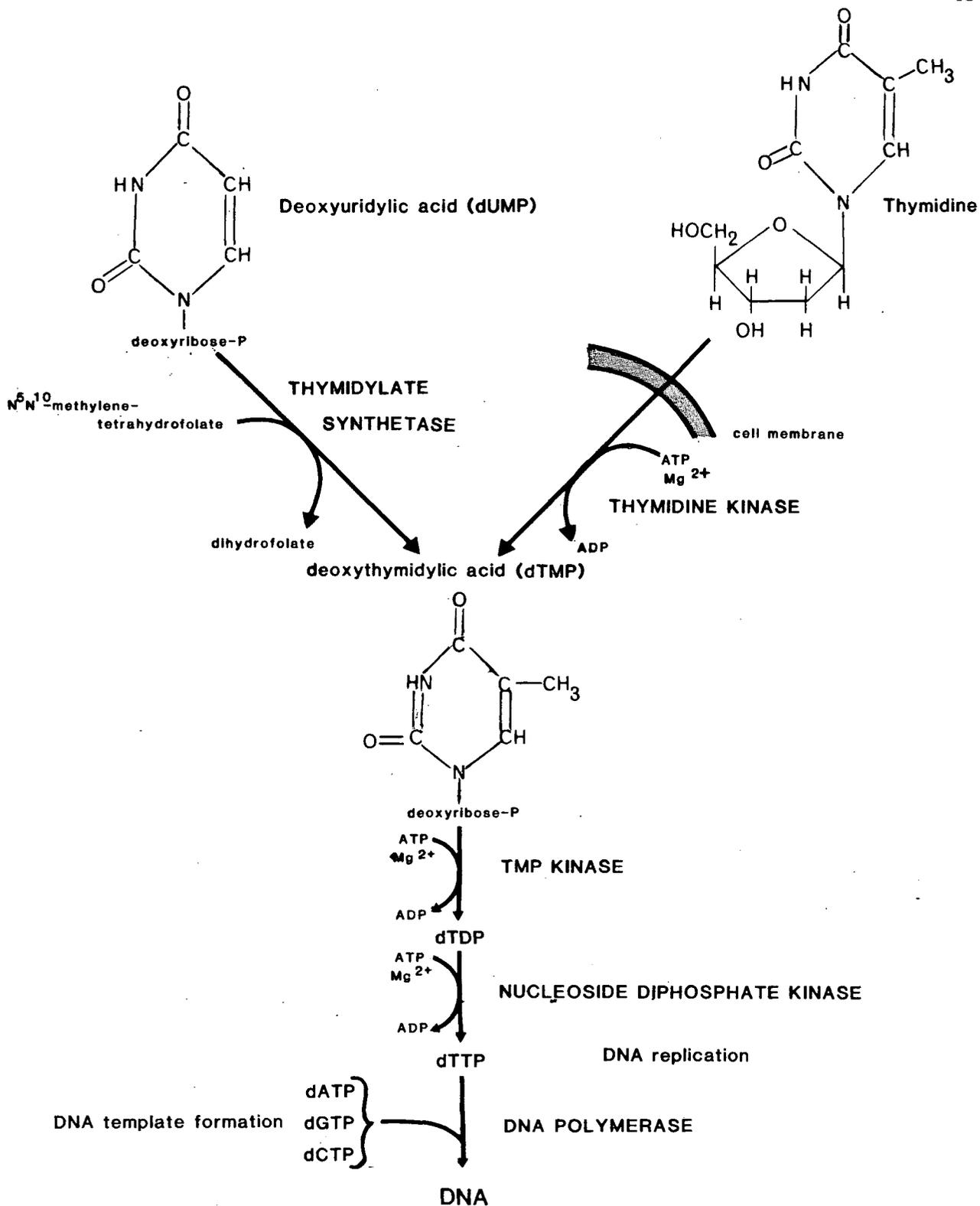
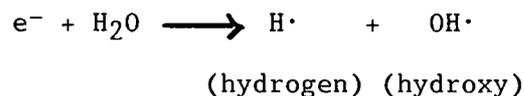


Figure 7. Schematic illustrating thymidine incorporation into DNA.

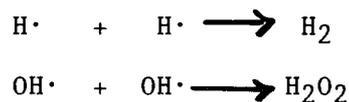
strand breaks. However, the contribution by ^3H transmutation to DNA strand breakage in mammalian cells is minor compared to the effects from the ^3H beta particle, though this latter effect may be important when mutations are considered.

Irradiation by emitted beta particles A beta particle from a decayed atom reacts with other portions of the molecule and/or with other nearby molecules by direct and indirect action. The beta particle ejected from ^3H decay produces a densely ionized column with an average track length of $1\ \mu\text{m}$ and with an average of 160 ionizations along the track. The ionizing effect of ^3H incorporation therefore, is not restricted to the molecular site but to an area of about $1\ \mu\text{m}$ in radius.

Beta particles produce damage primarily by the formation of free radicals which are neutral atoms or molecules having an unpaired electron. When ^3H incorporated into the DNA of a cell nucleus decays, the electron emitted reacts with the surrounding water to form two types of free radicals:



which recombine to yield



Organic free radicals are formed when organic molecules (RH) combine with the hydroxy free radical



Most of the free radicals formed have a short lifespan, generally less than 10^{-10} seconds. Since they contain unpaired electrons they are very reactive, and can oxidize or reduce the biological molecules within the cell (see review by Spinks and Marks, 1976).

In the presence of oxygen the emission of beta particles can create energy excited oxygen species known as singlet or triplet oxygen. When this process occurs, certain electrons in the oxygen atom are raised to higher energy or excited states. This excitation energy can be transferred from one part of a molecule to another, or between molecules, altering normal chemical bonds within the DNA helix (Peak, 1981; Piette et al, 1981; Houba-Herlin et al, 1982).

Biological effects of ^3H -thymidine incorporation

Chromosomal aberrations The first report on ^3H induced chromosome breaks was published in 1958 (Taylor, 1958). Many authors have described qualitative similarities between the effects of ^3H decay on chromosomes and those produced by external ionizing radiation such as X-rays. The production of chromosome aberrations in cultured mammalian cells by incorporated ^3H -thymidine has been studied in Chinese hamster cells (Dewey et al, 1965, 1967; Brewer and Olivieri, 1973) and in monkey cells (Hung et al, 1973). More recently these studies have been extended to human leukocytes (Vig et al, 1968; Vig, 1974; Bosian et al, 1977; Hori and Nakai, 1978) and fibroblasts (Nalarajan and Meyers, 1979). Some of the effects induced by ^3H decay include double and single strand breaks, base destruction, chromatid gaps, breakage of glycoside-phosphate ester linkages, chromatid interchanges, and cross linking. The efficiency of incorporated methyl ^3H -thymidine for producing DNA strand breaks has been calculated as 2.1 single strand breaks for 1 ^3H decay (Cleaver et al, 1972). In addition, the production of chromosomal aberrations by incorporated ^3H is an essentially linear function of the number of decays occurring in the labeled nucleus (Dewey et al, 1965, 1967).

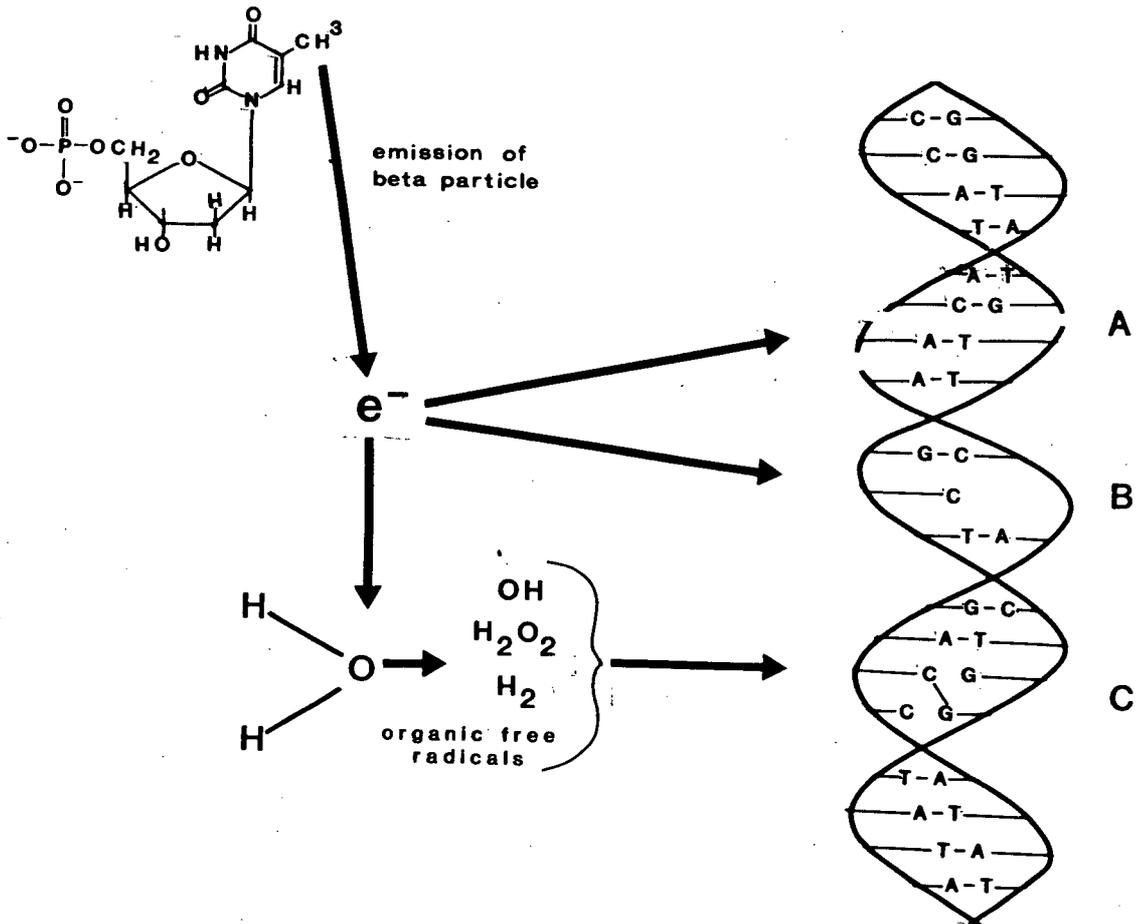


Figure 8. Schematic illustrating the most critical effects of ^3H -thymidine incorporation into DNA. Tritium decays to helium³ by emission of a beta particle which can react with other portions of the molecule or with other nearby molecules. The action of the beta particle may be direct or indirect, as with the interaction of the particle with a molecule of water resulting in the formation of free radicals. The most common of many types of DNA damage are shown here: A) single or double strand breaks in the DNA helix; B) loss of a base; C) cross-linking between two DNA strands.

Impairment of gene function Incorporation of ^3H -thymidine results in damage to the DNA which may impair several critical aspects of gene function. Single or double-strand cuts in the replicating forks of DNA may interfere with the unwinding and winding actions of replicating DNA (Cairns and Daverns, 1966). The induction of specific enzymes by bacteria was shown to be impaired after exposure to ^3H -thymidine (Rachmeler and Pardee, 1963). Other studies have demonstrated the mutagenic efficiency of ^3H -thymidine in bacteria (Person et al, 1976), in *Drosophila* (Olivieri and Olivieri, 1965) and in the mouse (Greulich, 1967; Bateman and Chandley, 1962).

Since the capacity of the mammalian cell to reproduce depends on the replicating ability of its DNA, such extensive damage to chromosomes would result in the inability of the cell to divide. Ultimately however, the damage to DNA that occurs as a result of ^3H decay may depend as much on the effectiveness of the repair processes as it does on the nature of the original lesion. When the cell attempts to rejoin chromosome breaks for example, the broken ends from one break may be joined incorrectly with those from another. Such breaks may be recombined to form various types of chromosome aberrations, such as translocations, inversions, rings, and other types of structural rearrangements (Upton, 1982). In this way even simple breaks may be lethal to the parent cell or its immediate progeny (Figure 8).

3) LONG TERM BONE MARROW CULTURES

The first successful culture system for the prolonged proliferation of hemopoietic stem cells was described by Dexter and his associates (1977) using murine bone marrow. The essential feature of this culture system was the prior establishment of a marrow derived adherent cell layer, upon which after 2 or 3 weeks a second inoculum of marrow cells could be seeded.

Maintenance of substantial numbers of hemopoietic cells was dependent on the formation of an adequate adherent layer in which numerous foci of large, lipid-containing cells were present. In turn the development of these fat cells was contingent upon a few selected lots of horse serum. However, Greenberger (1978a) reported that addition of corticosteroids could overcome the deficiency in unsuccessful lots of horse serum by increasing the number of lipid-containing cells. Furthermore, supplementation of serum with corticosteroids permitted the establishment of long term cultures with a single marrow inoculum, a procedure which had limited success previously.

Removal of the nonadherent cells on a weekly basis permitted their assay in conventional methylcellulose culture systems. In such a manner the continuous long term production of CFU-S (Dexter et al, 1977b) as well as committed progenitors of the granulocytic/macrophage, erythroid (Testa and Dexter, 1977; Gregory and Eaves, 1978) and megakaryocytic lineage (Williams et al, 1978b) could be demonstrated for several months. In addition the cumulative numbers of progenitors removed from the system at each weekly medium change may be greater than the number of cells present in the original inoculum suggesting de novo production of progenitors from the adherent layer (Eaves et al, 1983a). Further evidence for a progenitor:progeny relationship between the adherent and nonadherent cells is the demonstration that CFU-S in the adherent layer exhibit a higher self-renewal capacity than those in the growth medium (Mauch et al, 1980) and the demonstration of maintenance of a hemopoietically active non-adherent fraction with the removal at each week of all of the cells in that fraction (Greenberger et al, 1979).

Initial attempts to apply the principles of murine long term culture to human marrow were disappointing. Although recent improvements have permitted

the survival and perhaps limited proliferation of primitive granulocytic and erythroid progenitors in the nonadherent fraction of human cultures for several weeks (Greenberger et al, 1979; Gartner and Kaplan, 1980; Coulombel et al, 1983a) progenitor yields are not comparable to those obtained with the murine system, and most investigators would agree that existing culture conditions are not yet optimal. Nevertheless long term culture techniques have been usefully applied to marrow from normal subjects (Eaves et al, 1983; Coulombel et al, 1983a), from leukemic patients (Coulombel et al, 1983b) and from patients with myeloproliferative disorders (Powell et al, 1982; Coulombel et al, 1983b) and have offered new insights into normal and neoplastic hemopoietic mechanisms.

A) Description of the System

Upon initiation of mouse or human cultures there is an initial attachment and subsequent proliferation of marrow cells over a period of 2 to 3 weeks. A confluent multilayer is formed, composed of a variety of hemopoietic and nonhemopoietic cells of marrow origin (Dexter et al, 1977a; Coulombel et al, 1983a). Formation of "cobblestone" areas occur which are believed to represent foci of primitive hemopoietic cells (Figures 9 and 10) (Gartner and Kaplan, 1980; Greenberg et al, 1981). Above the adherent cells a layer of pseudo-adherent cells are found, with the largest concentration of these highly refractile cells often located just above the hemopoietic islands. These cells appear to be enmeshed in a viscous layer of material and are capable of only limited movement upon gentle agitation of the dish. They are not removed when the growth medium is changed.

Hemopoietic cells The progress of the long term culture can be assessed by monitoring the total cell and progenitor cell content of the nonadherent fraction. During the initial 2 to 3 weeks of culture there is a dramatic

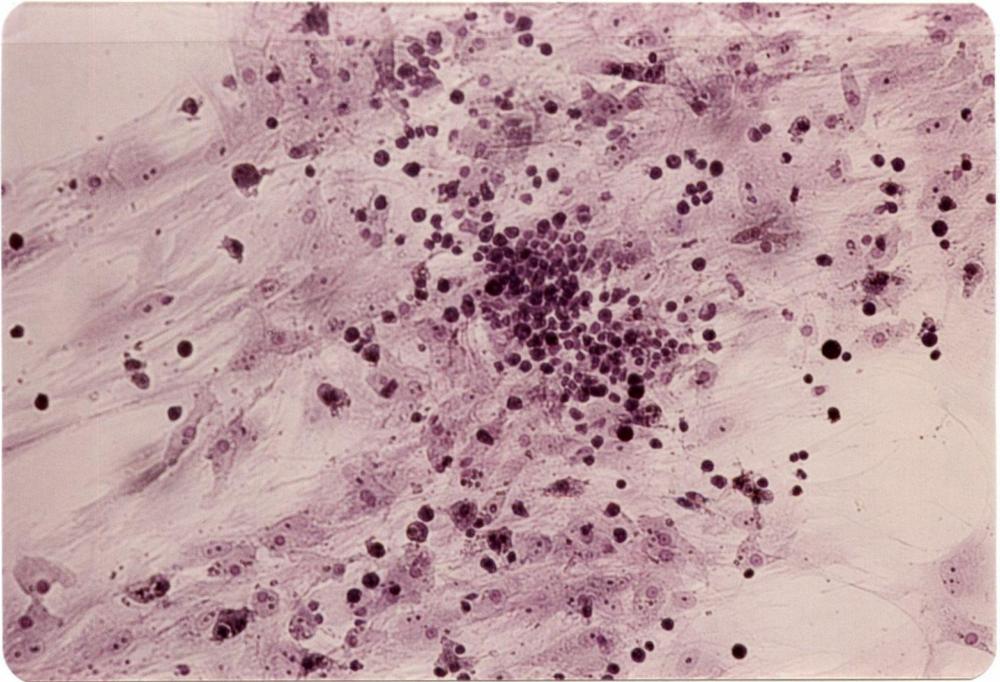


Figure 9. In situ staining of a putative cobblestone area in the adherent layer of a normal long term culture at 3 weeks of incubation (May-Grunwald-Giemsa staining, X160).

fall in the number of all cell types in the nonadherent fraction, followed by a plateau phase of several weeks duration when an equilibrium is reached between the rate of production and loss of the hemopoietic cells.

The majority of the supernatant population is composed of granulocytes in various stages of differentiation up to mature neutrophils and macrophages. The mature cells produced in human cultures are comparable to fresh peripheral blood granulocytes in physiological properties and retain many biological functions associated with bactericidal capacity in vivo (Greenberg et al, 1981).

Though primitive BFU-E can be detected in the supernatant for several weeks, morphologically recognizable erythroid cells rapidly disappear and are not found thereafter unless culture conditions are specifically modified to allow their production in vitro. Similarly, CFU-E and mature BFU-E can rarely be detected in the nonadherent fraction after 2 or 3 weeks (Dexter et al, 1978; Gregory and Eaves, 1978; Eaves et al, 1983)

B and T cell precursors at a primitive stage of differentiation are present in murine long term cultures, but as with erythropoiesis, culture conditions must be modified to permit production of their mature progeny in vitro (Schrader and Schrader, 1978; Jones-Villeneuve et al, 1980; Whitlock and Witte, 1982; Dorshkind and Phillips, 1983). The lymphoid potential of human long-term cultures has not yet been extensively investigated.

The development of a technique for the suspension of the adherent layer from human cultures for assessment in clonal assay systems has permitted the evaluation of their hemopoietic progenitor content (Coulombel et al, 1983a). Such studies have shown that after 4 weeks of culture the majority of BFU-E and CFU-C present in the cultures are located in the adherent layer. In addition more primitive progenitors, those capable of forming mixed colonies

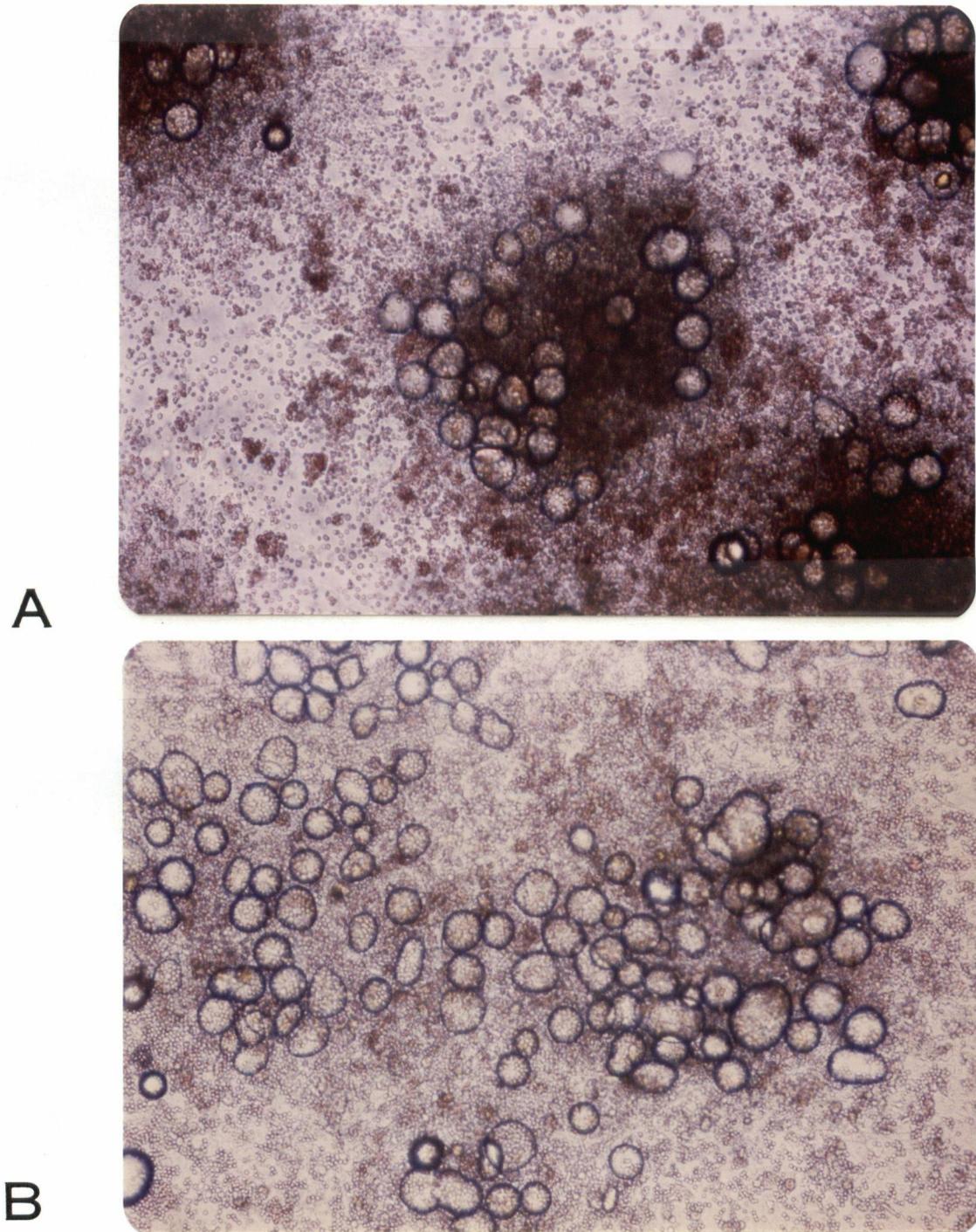
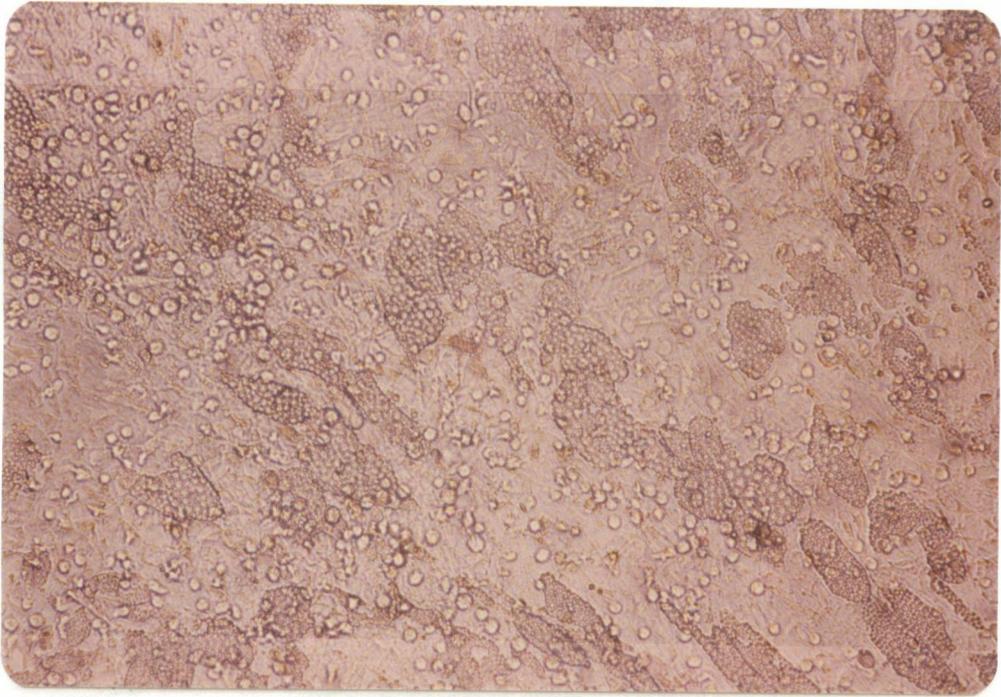


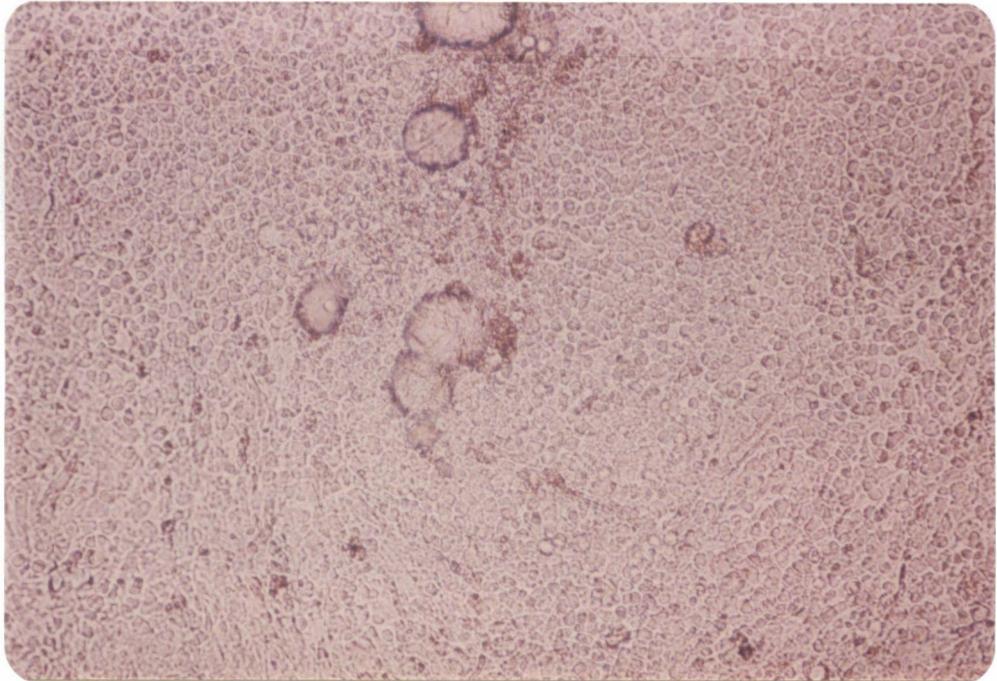
Figure 10. Morphological appearance of a PV long term culture adherent layer. These photographs were taken of the same culture dish at varying times after initiation.

A. Adherent islands present after 4 days of incubation (X80).

B. Another area of adherent islands 2 weeks after initiation. At this time the majority of the red blood cells have lysed (X80).



C



D

C. The adherent layer after 4 weeks of incubation showing the presence of numerous fat laden cells (X160).

D. The adherent layer 7 weeks after initiation. A few fat globules from an adherent island are still present (X160).

with granulocytic and erythroid elements (CFU-G/E) as well as CFU-C with very high proliferative capacities are located exclusively in the adherent layer after 2 to 3 weeks of culture. What determines such discrepancies in the growth of hemopoietic cells between the adherent and nonadherent fraction of long term cultures is not known, but may indicate a role for close interactions with stromal elements.

Stromal elements The composition of the stromal layer is controversial. In mouse cultures three main cell types were originally described on the basis of electron microscopy (Allen and Dexter, 1978): flat endothelial-like "pavement" cells, macrophages, and large lipid-containing cells. The presence of comparable cell types in human long term cultures has also been observed (Figure 11). In particular the appearance of fat-laden cells was seen in the initial attempts at culturing human marrow, and as in the murine system, an association of such cells with the more successful cultures was noted (Gartner and Kaplan, 1980).

Recently investigators have attempted to identify cell types in the adherent layer of murine cultures by delineating their secretion products. One laboratory reported finding collagen types I and III, and fibronectin, indicating the presence of fibroblasts, although no evidence for the presence of endothelial cell products was found by these authors (Bentley and Foidart, 1980; Bentley, 1982; Bentley and Tralka, 1982). However other laboratories have demonstrated the secretion of collagen types I, III, and IV, fibronectin, and lamimin in the adherent layer, suggesting the presence of both fibroblast and endothelial cell types in murine and human long term cultures (Castro-Malaspina et al, 1981; Keating and Singer, 1983; Zuckerman and Wicha, 1983; Zuckerman et al, 1983). Endothelial cells have been further characterized in human cultures as possessing a well developed submembranous

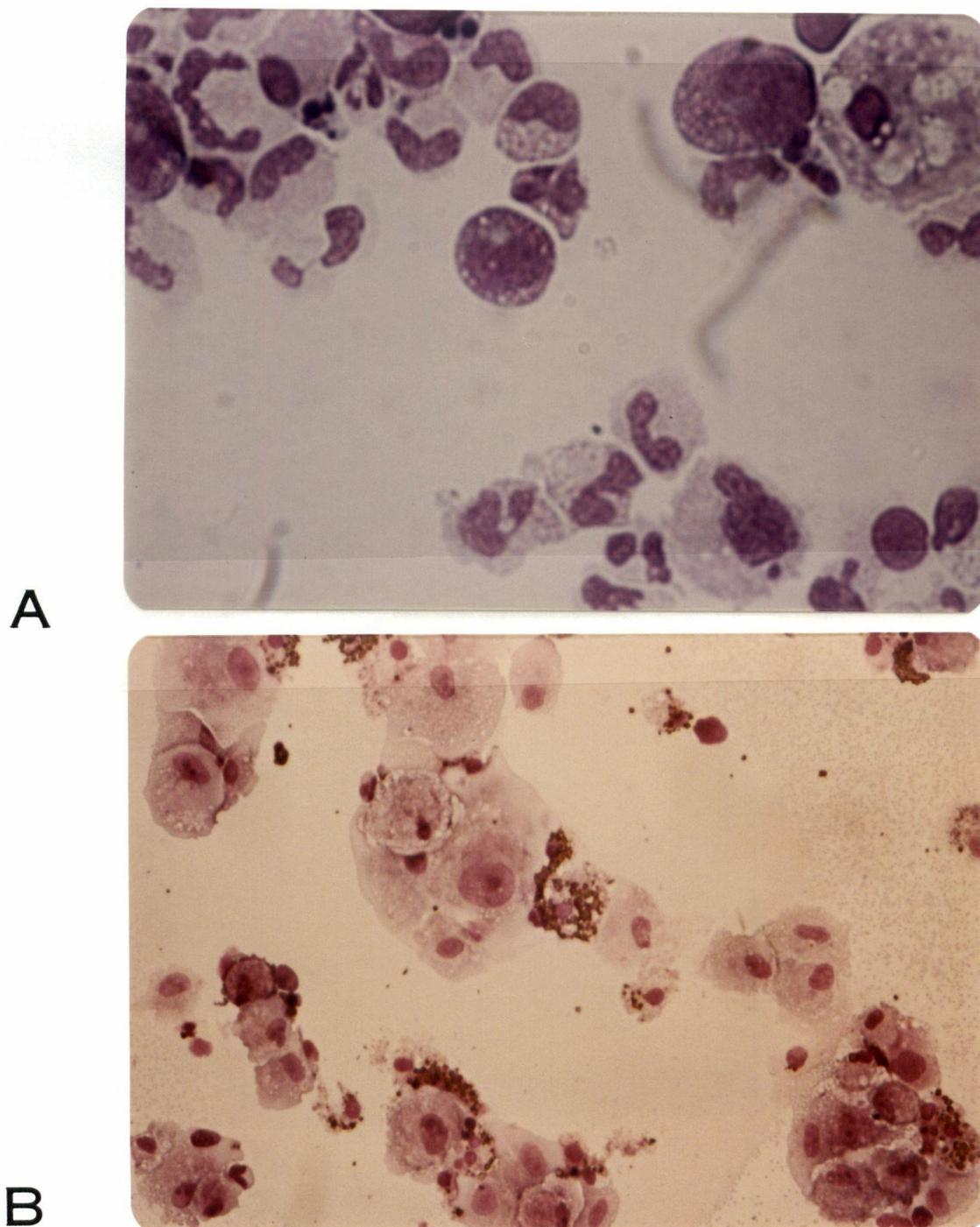


Figure 11. Cytospin preparations stained by the May-Grunwald-Giemsa procedure illustrating the morphological appearance of cells from a normal long term culture 3 weeks after initiation (X400).

A. Cells from the nonadherent fraction.

B. Cells from the adherent fraction, after detachment by trypsin.

microfilament layer, reactivity to Factor VIII antibody (Keating and Singer, 1983) and Weibel-Palade bodies, which are specific endothelial organelles (Allen, 1981). These cells may function in culture in a similar manner to their counterparts in vivo; mature granulocytes have been observed migrating through endothelial monolayers leading to an in vitro "transmural passage".

Macrophages, though hemopoietically derived are also part of the adherent layer and may be considered as part of the stroma in vivo. Cells which exhibit properties common to macrophages, such as esterase positivity and the presence of C_{3b} receptors are routinely observed in long-term cultures, though the F_C receptor, an essential feature of mononuclear phagocytes, was not demonstrable (Bentley and Foidart, 1980).

Recently another cell type, termed "blanket cells" have been characterized in long term cultures (Dexter et al, 1984). These are large, well spread alkaline phosphatase positive cells which are found overlaying numerous tightly packed macrophages and granulocytes in the cobblestone areas of the cultures, and may have a functional role in granulopoiesis.

D) The Role of the Adherent Layer

The importance of the adherent layer in the maintenance of hemopoiesis can be inferred from a number of studies. Stem cells are poorly maintained in siliconized cultured flasks where cell adherence is prevented. Cultures showing poor development of adherent cells are unsuccessful in producing stem cells for any extended period of time. Perhaps the most conclusive evidence for the role of adherent cells in in vitro hemopoiesis is given by experiments with genetically anemic mice (Dexter and Moore, 1977). The defect in the hemopoietically inefficient microenvironment characteristic of the S1/S1^d mouse may be reproduced in long-term cultures. Defective hemopoiesis results when normal, S1/S1^d stem cells are cultured on a S1/S1^d

adherent layer, although Sl/S1^d stem cells could be maintained on a normal adherent layer. These studies suggest that interactions between competent stem cells and an appropriate microenvironment are critical for the continuous in vitro proliferation of hemopoietic cells.

Cell to cell interactions The role of cell to cell interactions in murine long-term cultures was investigated by Bentley (1981) who placed hemopoietic cells in a diffusion chamber which in turn, was placed over a long term culture adherent layer along with free-floating cells. The rate of loss of CFU-S from the diffusion chamber where no direct interaction with stromal elements could occur, was greater than the rate of loss from the free floating population.

Successful hemopoiesis in vitro is correlated with the presence of large cobblestone areas where granulocyte development occurs. All the stromal cell types previously described are found here in intimate contact with the developing hemopoietic cells. Junctional membrane complexes between the hemopoietic and stromal elements may be indicative of intercellular metabolic cooperation. Extensive interdigitative coupling of blast cells to macrophages has also been observed (Lambertsen, 1984). In addition, numerous coated vesicles present in the closely opposed regions of membrane between the developing hemopoietic cells and macrophages are suggestive of the short-range release and receptor-mediated endocytosis of regulatory factors (Allen and Dexter, 1982). Such intimate opposition of large areas of membrane also occur extensively between adjacent macrophages across the entire cobblestone area.

Though normal long-term cultures do not support the further differentiation of erythroid progenitors, the addition of anemic mouse serum or Ep coupled with a mechanical stimulus will result in full erythroid

maturation (Allen and Dexter, 1982). Modifications in the adherent layer occur with the induction of erythropoiesis, and erythroblastic islands consisting of synchronously maturing cohorts of erythroid cells in close association with a central macrophage appear. The developing erythroid cells have large areas of closely opposed membrane, gap junctions, and possible reciprocal vesicular activity, indicative of cell to cell cooperation. Similar associations between clusters of normoblasts and erythrocytes with macrophages have been observed in human long term cultures (Hocking and Golde, 1980).

Regulatory factors Despite the continuous proliferation of granulocytic cells in vitro initial studies indicated that CSF was not present in long term murine or human cultures (Dexter et al, 1977b; Toogood et al, 1980). Furthermore the addition of anti-CSF antiserum on a weekly basis to long term cultures did not reduce the production of granulocytes or other hemopoietic cells (Dexter and Shadduck, 1980). A marked decrease in granulocyte production occurred when a crude source of CSF was added (Dexter et al, 1977b), although more recent experiments have shown that this effect could be attributed to impurities in the CSF preparation as the addition of a more purified CSF had no effect (Dexter and Shadduck, 1980; Williams and Burgess, 1980).

Other studies have provided evidence for CSF production in long-term cultures by demonstrating colony formation by fresh hemopoietic cells immobilized in agar overlaying the adherent layer (Gualtieri et al, 1982). The observation of an inverse relationship between the number of hemopoietic cells and the levels of CSF in long-term cultures led to the suggestion that a local regulatory mechanism exists resulting in feedback inhibition of CSF production (Gualtieri et al, 1984) or the consumption of CSF by differentiating myeloid cells (Gualtieri et al, 1982; Heard et al, 1982).

The development of a sensitive radioimmunoassay for CSF has permitted its detection in virtually all long term media examined (Shaddock et al, 1983). In addition, the removal of inhibitory substances, or the concentration of supernatant has resulted in the routine detection of biological activity in media previously considered inactive (Shaddock et al, 1983). Recently a number of cell lines have been generated from the adherent layer which are active producers of CSF providing further evidence that CSF is involved in the control of cell production in this system (Harigaya et al, 1981; Cronkite et al, 1982; Lanotte et al, 1982).

The production of erythropoietic factors in long term culture is less well documented. The presence of Ep is not demonstrable in this system. Attempts to measure levels of burst promoting factor were not successful, as conditioned media from the cultures was found to be strongly inhibitory to burst formation (Eliason et al, 1982). A lack of BPA in this system might be suggested by the absence of erythroid development. However, this does not proceed when BPA is added to the cultures. On the other hand, subcultures of adherent cells are capable of producing BPA, which has led to the hypothesis that in the intact system other cells in the culture may block BPA production (William et al, 1979). The effect of anemic mouse serum in supporting erythropoiesis in these cultures (Dexter et al, 1981) could result from inhibition of those cells which in turn prevent the production of BPA. The demonstration that the removal of certain cell types from the cultures can greatly increase the yield of BFU-E provides additional evidence for the presence of inhibitory cells (Williams et al, 1979).

In an analysis of stem cell cycling in murine long term cultures Toksoz and his associates (1980) provided evidence that the adherent layer produces factors that can modify the proliferative activity of stem cells. In earlier

experiments utilizing ^3H -thymidine (Dexter et al, 1977b) these investigators had reported that CFU-S proliferative activity in long term cultures follows a cyclic pattern related to the feeding regime. Within 1 to 2 days after replacement of the growth medium and demipopulation of the cultures a high proportion of the CFU-S were in S phase. However, within 4 to 7 days after the cultures were fed, the proportion of CFU-S in active cell cycle progressively decreases until a majority are quiescent. Following a further feed the pattern of stimulation of cycling activity and eventually reversion to a noncycling state could be repeated. During this entire period CFU-C remained in a constant high cycling state. Subsequently these authors demonstrated that this cyclic pattern of CFU-S proliferation was correlated with the release into the medium of stimulatory and inhibitory factors similar to those described previously (Lord et al, 1976, 1977) in normal and regenerating bone marrow. When CFU-S were in active cell cycle one day after feeding the cultures, the concentration of the stimulatory material increases relative to the inhibitor, while the reverse situation is found several days later when the CFU-S are quiescent. In addition, the proliferative activity of CFU-S, whether in an active or noncycling state could be modified by the addition of the appropriate factors.

In further experiments these authors concluded that the addition of fresh media or the absolute cell number was not the primary stimulus for the production of these factors (Dexter et al, 1980). Simple mechanical agitation or the replacement of spent media could reproduce the cyclic pattern of CFU-S proliferation. This finding led to the formulation of a model in which the proximity of CFU-S to the adherent layer is the critical stimulus. A stem cell within the adherent layer could signal its presence leading to the production of inhibitor. Removal of the stem cell, by

mechanical means, or by differentiation, death, or migration would result in stimulator production. It is of interest that in this system the proliferation of CFU-S results in stem cell differentiation, not self-renewal, as there is no increase in the total number of CFU-S in the culture.

Role of the extracellular matrix (ECM) Interactions between parenchymal cells and the extracellular matrix have a central role in differentiation in a variety of cell systems (Grobstein , 1975). Recent interest therefore has focussed on the possibility that hemopoietic stem cell differentiation may be influenced in a similar manner by interactions with connective tissue components. Examination of the distribution of glycosaminoglycans (GAGs) in long-term cultures has shown a constant pattern of sulphated GAGs associated with the adherent layer, where heparan sulphate is a main component, and the medium, where chondroitin sulphate predominates (Spooner et al, 1983). Addition of β -D-xylosides resulted in a dramatic increase in chondroitin sulphate content in the growth medium and had a marked stimulatory effect on hemopoiesis in suboptimal cultures by increasing the porportion of CFU-S in S phase.

Other investigators have demonstrated that high concentrations of chondroitin sulphate and heparin sulphate could block the response of CFU-E to the addition of Ep while other sulphated GAGs had no effect (Ploemacher et al, 1978). On the other hand, low concentrations of chondroitin sulphate had a stimulatory effect on CFU-E plating efficiency. A comparable effect on granulocytic colony formation was not observed.

Other investigators have investigated the role of fibronectin in cell adhesion in long term cultures. Fibronectin has been detected on various adhesion sites, including substratum attachment surfaces of adherent cells, between fibroblastoid elements and at sites of interactions between hemopoietic cells and adherent cells (Bentley and Tralka, 1982).

4) THE MYELOPROLIFERATIVE DISORDERS

The term "myeloproliferative disorders" (MPD) was introduced by Dameshek (1951) to describe a group of closely related syndromes - polycythemia vera (PV), essential thrombocytosis (ET), chronic myeloid leukemia (CML) and myeloid metaplasia with myelofibrosis - all of which share a number of clinical and pathological features. The most outstanding unique characteristic of each of these diseases is the elevation of a single hemopoietic cell lineage (i.e. RBC in PV, platelets in ET, granulocytes in CML). However, the common involvement of the lineages suggested that each originated from the disordered growth of a pluripotent stem cell, although conclusive evidence for the existence of such a cell was not obtained until ten years later. Evidence that these diseases represent clonal neoplasms was also established at about the same time with the consistent demonstration in CML patients of the Philadelphia (Ph^1) chromosome in multiple hemopoietic cell lines (Figure 12) (Nowell and Hungerford, 1961; Whang et al, 1963; Golde et al, 1977).

Another approach to the study of clonality in the MPD has made use of a different genetic marker system, the isoenzymes of glucose-6-phosphate dehydrogenase (G6PD). The gene for G6PD is on the X chromosome. Since one X chromosome in each cell in women is inactivated at random early in embryogenesis and the choice is then fixed in all subsequent progeny, most tissues in G6PD female heterozygotes are composed of equal numbers of cells synthesizing one or the other isoenzyme type (Lyon, 1961). The isoenzyme product of the usual gene Gd^A and of some variants, such as Gd^B , may be separated by electrophoresis. Using this marker system only one isoenzyme type was seen in the circulating erythrocytes, granulocytes and platelets of G6PD heterozygotes with Ph^1 negative and Ph^1 positive CML (Fialkow et al,

1967, 1977; Douer et al, 1981), PV (Prchal et al, 1976), ET (Fialkow et al, 1981) and myeloid metaplasia with myelofibrosis (Jacobson et al, 1978) even though in each case mosaicism was demonstrated in their skin fibroblasts. When individual hemopoietic colonies were tested, direct evidence of early progenitor involvement in these neoplasms was also obtained (Aye et al, 1973; Moore and Metcalf, 1973; Singer et al, 1979; Dube et al, 1981). Recently the demonstration of clonality in multilineage colonies has confirmed the involvement of pluripotent cells (Douer et al, 1981; Dube et al, 1984a). Nevertheless, the mechanism responsible for the selective growth advantage of the progeny of the abnormal clone remains obscure.

Typically, the MPD exhibit a prolonged chronic steady state which may persist for a decade or more with minimal intervention, although progression to an acute leukemia (blast crisis) commonly occurs within 3 to 5 years in patients with CML. Despite the extensive number of progeny produced by the single abnormal pluripotent stem cell, essentially normal, functional mature cells are formed. However, a number of hematological abnormalities may be found to a varying degree in each disease. These include abnormalities of red cell, leukocyte and platelet counts, extramedullary hemopoiesis, abnormal leukocyte alkaline phosphatase values and elevations in serum B₁₂ and B₁₂ binding capacity. In addition, a number of investigators have shown the presence of erythroid progenitors capable of terminal differentiation in vitro in the absence of added Ep in CML (Eaves and Eaves, 1979), PV (Prchal and Axelrad, 1974; Eaves and Eaves, 1978) and ET (Prchal and Axelrad, 1974; Eaves et al, 1983b). Though diagnostic for PV (Eaves et al, in press) the expression of Ep independent growth is much more variable in CML and ET but still suggests a common biological abnormality in all three disorders (Eaves et al, 1980).

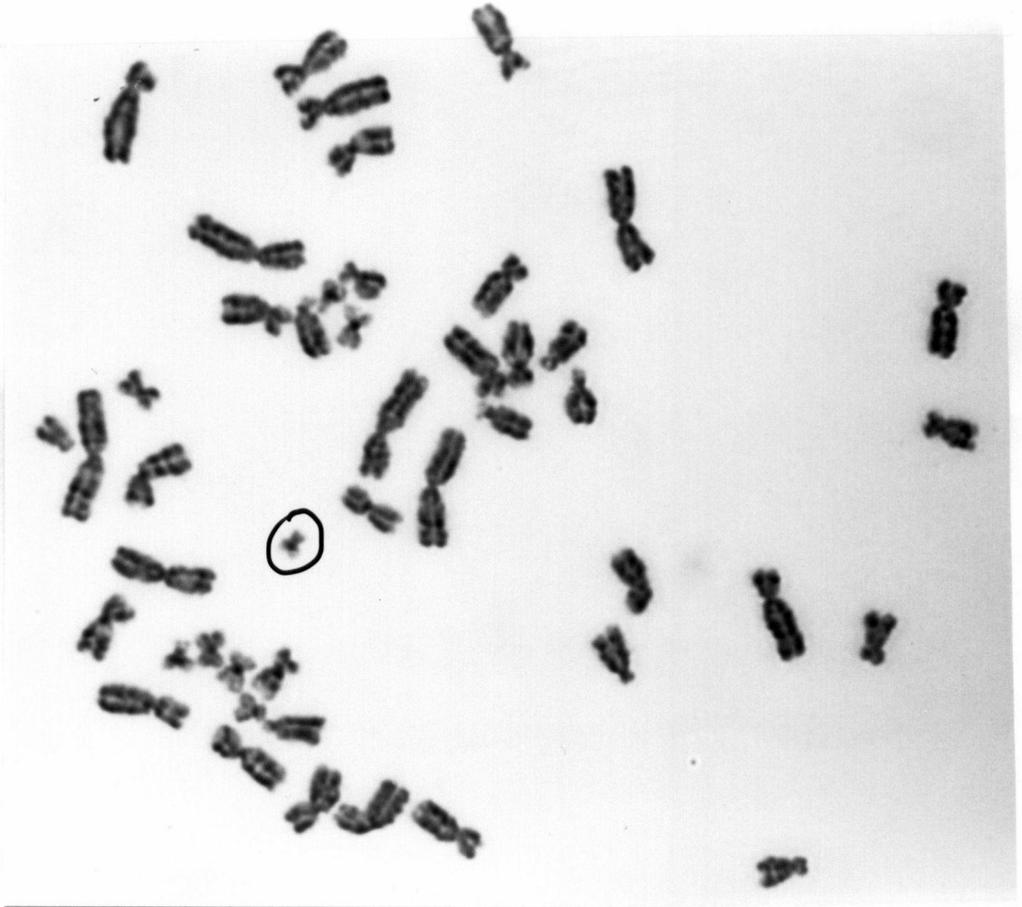


Figure 12. Metaphase chromosomes from a CML patient. In this particular case the Ph¹ chromosome (circled) was formed by the transfer of part of chromosome 22 to chromosome 16. This metaphase was obtained from an erythroid colony in methylcellulose culture established with cells from the nonadherent fraction of a 5 week old reconstituted long term blood culture.

A) Chronic Myeloid Leukemia

The most outstanding clinical feature of CML is the elevation in the number of circulating white blood cells (WBC) which may rise to as much as 100 times normal values. In addition to mature granulocytes, immature forms are also found. The age of onset of CML is variable, but the disease is most frequent above the age of 40. Typical symptoms at presentation include fatigue, low grade fever, weight loss, pallor, and discomfort due to an enlarged spleen or liver (Wintrobe, 1976). Laboratory studies indicate that all stages of the neutrophilic series, from myeloblasts to segmented neutrophils are present, usually in normal ratios. Anemia is often present at diagnosis. Approximately half of the patients will have some degree of thrombocytosis, and in some cases this may be severe with platelet counts of over $10^6/\text{mm}^3$. Most of these symptoms can be reduced or eliminated in the chronic phase by treatment with cytotoxic agents, such as busulfan or hydroxyurea. CML usually terminates in a blast crisis with many of the characteristics of acute myeloid leukemia. The onset of blast crisis in relation to time of diagnosis is quite variable, but most series indicate a median time to transformation of about 3 years. Despite new and more aggressive therapeutic modalities this median time has not changed in 50 years (Minot et al, 1924). During blast crisis the WBC may rise to extreme levels, but the more important feature is the rapid accumulation of immature blasts. These appear to resemble immature members of the neutrophil series in many cases although all lineages may be involved including cells of the B lymphoid series. As the blast phase proceeds normal blood elements decrease in number, recurrent infection and hemorrhage occur, and these are usually the cause of death. Remissions are rare, and even when they occur tend to be of short duration, seldom lasting more than 6 months. There are no well

documented cases of cure with chemotherapy, though some success has been obtained with bone marrow transplantation (Armitage et al, 1984).

Chromosomes in CML The majority of CML patients (80-95%) have a unique chromosomal marker, the Philadelphia chromosome (Ph^1) (Nowell and Hungerford, 1960). Though initially described as an abnormally small G group chromosome, the development of banding techniques enabled Rowley (1973) to identify the Ph^1 chromosome as a balanced translocation between the long arm of chromosome 9 and the long arm of chromosome 22 - $t(9;22)(q34;q11)$. Most patients will have the classic Ph^1 chromosome as the sole cytogenetic abnormality at least in the chronic phase of their disease. Occasionally, a complex translocation involving several chromosomes, particularly no. 17 may be found (Rowley, 1980). An atypical Ph^1 chromosome does not appear to influence survival as the clinical course of these cases does not differ significantly from patients with the typical 9;22 translocation (Sandberg, 1980).

Additional changes in karyotype occur at blast crisis in about 75-80% of all Ph^1 positive patients. These secondary aberrations are superimposed on the Ph^1 cell line, and are nonrandom. In order of frequency about 80% of the cases will involve one or more of the following: 1) double Ph^1 ; 2) additional no. 8; 3) abnormalities of 17q; 4) additional no. 19; 5) additional no. 21 (Rowley, 1976; Pearson et al, 1983). In some patients these additional chromosomal aberrations may be present weeks or months before blast crisis suggestive of an "accelerated phase" of progressive genetic change finally leading to disruption of normal differentiation potential (Sandberg, 1980).

New interest in the relationship of the Ph^1 chromosome to the abnormal control of hemopoietic differentiation in CML has arisen with the demonstration that the translocation of the human cellular homologue of the transforming sequence of Abelson murine leukemia virus (c-abl) from

chromosome 9 to a specific region on chromosome 22q (de Klein et al, 1982) is a consistent feature in Ph¹ positive cells, even when more complex translocations are involved (Bartan et al, 1983). Subsequently it was shown that the fusion region of chromosome 9 to chromosome 22 was contained within a 5.8 kilobase (kb) segment. This region on chromosome 22 was designated bcr (breakpoint cluster region) (Groffen et al, 1984). The translocation fuses the c-abl oncogene to a bcr gene on the Ph¹ chromosome (Heisterkamp et al, 1985; Shtivelman et al, 1985) resulting in the formation of a hybrid mRNA of about 8 kb (Collins et al, 1984; Gale and Canaani, 1984). In turn, the novel mRNA codes for an altered 210K molecular weight polypeptide which has been identified in the leukemic cells of essentially all CML patients and CML cell lines examined (Collins et al, 1982; Blick et al, 1984; Konopka et al, 1984). The bcr-abl polypeptide from CML cells was shown to possess tyrosine kinase activity, unlike its normal 145K counterpart (Konopka et al, 1984; Konopka et al, 1985).

Differentiation potential of the CML stem cell The Ph¹ chromosome has been found in 90-100% of bone marrow metaphases in CML patients early on, indicating its presence in both granulocytic and erythroid lineages (Whang-Peng et al, 1963). The presence of the marker in metaphases of cells capable of hemoglobin synthesis as measured by radioactive iron uptake was more direct evidence for erythroid cell involvement in the abnormal clone (Rastrick et al, 1968). In addition metaphases of cells from individual erythroid (Dube et al, 1981), granulocytic (Chervenick et al, 1971) and macrophage (Golde et al, 1977) colonies grown in vitro have also demonstrated the Ph¹ chromosome. Megakaryocytic involvement in the neoplastic clone has been confirmed by the presence of the Ph¹ marker in tetraploid and octaploid bone marrow cells (Tough et al, 1963; Whang-Peng et al, 1968).

Early reports indicated that the Ph¹ chromosome was restricted to cells of the myeloid lineages. However, a minority of patients with CML progress to a blast phase in which the predominant cell types have lymphoid characteristics, including a cellular content of a unique enzyme, terminal deoxynucleotidyl transferase (TdT) previously identified in the lymphoblasts of patients with acute lymphoblastic leukemia (ALL) (McCaffrey et al, 1973, 1975). These blast cells may share certain antigenic features with ALL lymphoblasts (Janossy et al, 1976), contain cytoplasmic immunoglobulin (LeBien et al, 1979) and demonstrate immunoglobulin gene rearrangements typical of pre-B cells (Bakhshi et al, 1983). Relatively early it was postulated that a myeloid-lymphoid stem cell was the site of the lesion in CML (Boggs, 1981). Considerable evidence has now been presented for the involvement of the B cell lineage even in chronic phase CML. The Ph¹ chromosome has been detected in cells which also demonstrated the presence of surface immunoglobulin, a marker for B cells (Bernhiem et al, 1981). Cytogenetic studies of Epstein Barr virus (EBV) transformed lymphoid cell lines from a CML patient heterozygous for G6PD isoenzymes has also demonstrated the presence of the Ph¹ chromosome in some of these (Martin et al, 1980). In other studies Ph¹ positive B cells were found in about 25% of CML patients after transformation with EBV (Nitta et al, 1985).

The possible involvement of T lymphocytes in the malignant clone remains controversial. PHA stimulated T lymphocytes have consistently been found to be cytogenetically normal even in a patient in chronic phase CML of long duration (Kearney et al, 1982; Nitta et al, 1985). Isoenzyme studies have shown T lymphocytes to be polyclonal, whereas in the same patient the B lymphocytes demonstrated only one isoenzyme type (Fialkow et al, 1978). On the other hand, the presence of Ph¹-positive T lymphocytes in chronic phase

CML has been reported by two groups (Shabtai et al, 1980; Itani and Hashino, 1982). In addition, several examples of lymphoblastic transformation of CML have been reported in which T cell involvement was established by the presence of T cell specific surface antigens on Ph¹-positive cells (Janossy et al, 1978; Hernandez et al, 1982; Griffin et al, 1983; Herrman et al, 1984; Jacob and Greaves, 1984). Moreover, Ph¹ positive T lymphoblasts have been demonstrated in the lymph nodes of CML patients, without detectable preceding or concurrent lymphoblastic transformation in the marrow (Palutke et al, 1982; Jacob and Greaves, 1984). Although the majority of investigators have not been able to demonstrate T cell involvement in CML the possibility remains that since T lymphocytes are very long lived cells, they may be progeny of the abnormal stem cell which antedate the development of the malignant lesion in that cell.

Residual normal stem cells in CML Since the goal of chemotherapy in CML is eradication of the abnormal clone, the question of residual normal hemopoiesis in these patients is of considerable therapeutic importance. Early studies indicated the presence of the Ph¹ chromosome in all dividing hemopoietic cells examined, even in treated patients whose counts have been returned to normal levels. In addition, application of the in vitro colony assay system demonstrated the all CFU-C had the same isoenzyme type as the abnormal clone in several patients initially studied in this way (Fialkow et al, 1978b; Singer et al, 1979b). In other studies however, some Ph¹-negative granulocyte colonies were seen in 2 out of 4 CML patients (Chervenick et al, 1971). More recently, in serial cytogenetic studies, 10 of 41 Ph¹-positive CML patients demonstrated Ph¹-negative cells in their early chronic phase though the percentage of such cells decreased during the course of the disease and were absent in the acute phase (Sonia and Sandberg, 1978). In

in vitro studies on the progeny of colony-forming cells also revealed the presence of Ph¹-negative progenitors in a number of CML patients whose WBC counts were within normal limits (Dube et al, 1984a). The presence of Ph¹-negative cells in Ph¹ positive CML has been correlated with increased survival time (Sakurai et al, 1975) though this has been disputed by others (Sokal, 1980). A more aggressive chemotherapeutic protocol has resulted in the appearance of Ph¹-negative cells in two other studies (Cunningham et al, 1979, Goto et al, 1982) but in each case the reduction in Ph¹-positive cells was temporary, and did not affect the survival of the patients. In one patient heterozygous for G6PD alleles, the emergence of Ph¹-negative cells was paralleled by the return of both isoenzyme types, indicating that the Ph¹-negative cells were not clonal in origin (Singer et al, 1980a). More recently, the use of long term marrow cultures has offered a more sensitive assay for residual normal cells in CML. Coulombel and her associates (1983b) have shown that when marrow cells from newly diagnosed Ph¹ CML patients were placed in culture, in the majority of cases a previously undetectable population of chromosomally normal hemopoietic cells of all myeloid lineages could be demonstrated within two to four weeks. The number of Ph¹ positive cells rapidly declined but Ph¹-negative progenitors could be maintained for 8 to 12 weeks in such a culture system. In order to determine if these Ph¹ negative progenitors were nonclonal, marrow cells from a mosaic Turner's syndrome patient with CML (46,XX/45,X,Ph¹) were placed in long term culture (Dube et al, 1984b). All the metaphases examined from fresh marrow preparations and in vitro granulocytic and erythroid colonies obtained from marrow progenitors were 45,X,Ph¹, while granulocytic colonies from the adherent layer of the long term cultures yielded only 46,XX metaphases. Since the patient had been diagnosed 5 years previously these studies provide

additional evidence of the long term persistence in vivo of normal, nonclonal progenitors in at least some CML patients.

Cell culture studies in CML Numerous investigators have demonstrated a large increase in the number of CFU-C in the blood of patients with CML (Moore et al, 1973; Goldman et al, 1974; Eaves and Eaves, 1979). A relationship has been noted between the increase in blood CFU-C and the WBC count (Goldman et al, 1974, Olofsson and Olsson, 1976). The CFU-C concentration usually returns to normal when the WBC is brought down to within normal limits. In the marrow, the CFU-C concentration is more variable, with most investigators reporting a modest increase in CFU-C number (Goldman et al, 1974; Eaves and Eaves, 1979). In some CML patients therefore, the concentration of CFU-C per 10^5 cells in the blood exceeds the proportion in the marrow, unlike the normal situation. The morphology of the colonies produced in vitro during the chronic phase, however, appears to be normal, and the maturity and distribution of the cell types is typical of that seen in normal subjects. When blast crisis intervenes fewer normal sized colonies are seen. In some cases large numbers of small (<50 cell) colonies, composed of poorly differentiated cells as seen in acute leukemia, may be produced (Moore et al, 1973, Goldman et al, 1980)

Eaves and Eaves (1979) reported that large increases in the CFU-E and BFU-E compartments also accompany, and parallel, the increase seen in CFU-C. Thus, the ratio of BFU-E:CFU-C numbers in the circulation of CML patients is constant and does not differ substantially from that seen in normal individuals (Eaves et al, 1980). This suggests that the large increase in mature granulocytes is not due to a preferential "channeling" of progenitors into the granulocytic pathway at the level of the pluripotent stem cell, but rather lack of terminal control of granulopoiesis post CFU-C.

The cell cycle characteristics of CFU-C are altered in CML. A number of investigators have noted that fewer marrow CFU-C are in S-phase in chronic phase CML compared to normal controls (Moore et al, 1973; Rickard et al, 1979; Singer et al, 1981) suggesting that a greater than normal number of CFU-C are in a noncycling state. Restoration of the cell cycle characteristics of CFU-C to normal was achieved after treatment (Moore et al, 1973). During blast phase, the proportion of CFU-C in S phase was found to be increased over chronic phase values, despite an increasing leukocytosis (Moore et al, 1973; Rickard et al, 1979).

Pluripotent progenitors (CFU-GEMM) in chronic phase CML were also found to show alterations in cell cycle activity. In the blood and marrow of normal subjects these primitive progenitors are quiescent (Fauser and Messner, 1982a), but in CML CFU-GEMM were shown to be in active cell cycle (Messner et al, 1980; Lepine and Messner, 1983). Unlike the previous studies of CFU-C, the proliferative rate was not modulated in treated patients, thus indicating that such alterations in cycling kinetics may be fundamental to the disease process. An increase in the frequency of circulating CFU-GEMM, up to 600 fold over normal values, was noted by some investigators (Hara et al, 1981, Hibbin et al, 1983) but not by others (Messner et al, 1980; Lepine and Messner, 1983).

Regulatory abnormalities Defects in negative feedback regulation have been suggested as a possible mechanism for the excess myelopoiesis seen in CML. For instance, colony formation by CFU-C from CML patients is not inhibited by concentrations of PGE₁ that inhibit normal CFU-C and this altered sensitivity persisted even after treatment (Pelus et al, 1980). In another study, neutrophils from CML patients were defective in production of colony inhibiting activity (CIA), subsequently identified as lactoferrin,

which decreases production and release of CSA from monocytes and macrophages (Broxmeyer et al, 1977). In addition, the CSA producing cells from CML patients were less sensitive than normal cells to inhibition with low concentrations of lactoferrin obtained from normal neutrophils. However, contradictory results were obtained by other investigators (Moberg et al, 1978).

CFU-C colony formation in CML is comparable to normal colony formation in its strict requirement for CSF (Moore et al, 1973; Metcalf et al, 1974) although differences have been noted in the threshold sensitivity of the CFU-C. Levels of CSF in the serum and urine of chronic phase CML patients are normal or increased (Moore and Robinson, 1974), while in blast phase no activity is detectable (Moore et al, 1973; Golde et al, 1974). The colony stimulating activity of peripheral blood leukocytes from CML patients was found to be lower than normal by some authors (Goldman et al, 1974) but not by others (Moore et al, 1973). When abnormal, the colony stimulating activity returned to normal following treatment, suggesting that the decrease in CSF production by leukemic cells was due to the inhibitory effects of the large numbers of circulating granulocytes. Marrow samples from most patients were reported to have normal colony stimulating activity, but a few patients had consistently higher levels of activity (Bianchi Scarra et al, 1981). However, because of the lack of precision in these assessments and the fact that a number of different molecules are now known to be active in CFU-C colony stimulating assays, these studies are difficult to interpret.

In summary, CML is a clonal disorder, with a prominent leukocytosis, terminating in a blast phase and subsequent death. The Ph¹ chromosome is a consistent finding in the large majority of these patients. Other chromosomal abnormalities may also be seen, particularly in blast phase, but

the contribution of any of these to the clinical course of the disease has yet to be established. Recent evidence indicates that residual normal stem cells are found in a large porportion of CML patients, though their existence can often be documented only by in vitro assays. A number of other anomalies are seen on examination of the growth characteristics of CML cells. These included increases in progenitor cell number, differences in cell cycle activity, and possibly the altered production of some regulatory factors. At the present time, however, the role of each of these findings in the pathogenesis of CML remains unclear.

B) Polycythemia Vera (PV)

The outstanding feature of PV is an absolute increase in the red cell mass, which is often associated with simultaneous or sequential cytopathological proliferative changes in the marrow resulting in a panmyelosis of varying degree. The patient initially presents with symptoms resulting from increased blood volume and hyperviscosity. Cerebrovascular accidents or myocardial infarction may result if the disease is not treated. The usual age of onset of PV is in the middle or later years with a peak in the fifth or sixth decade (Wintrobe, 1976).

The chronic phase of PV may last anywhere from 5 to 20 years. Therapy during this period may include phlebotomy, and/or treatment with radioactive phosphorus or cytotoxic drugs such as busulfan. With such treatment many of the symptoms usually disappear and the circulating cell mass may be brought down to within normal limits. With time the disease may evolve into a spent phase, characterized by extramedullary hemopoiesis, an increasing degree of anemia, and the development of myelofibrosis (Ward and Block, 1971). This phase has been called post polycythemic myeloid metaplasia (PPMM) and varies in frequency from 7-30% of cases (Silverstein, 1976). In 1-30% of patients,

the disease terminates in leukemic changes typical of acute myeloblastic leukemia with progressive anemia, thrombocytopenia and the appearance of blast cells (Ellis et al, 1975; Wasserman, 1954, Glasser and Walker, 1969). The contribution of therapy to the evolution of PV to acute leukemia is now well documented (Berk et al, 1981).

Cytogenetics Though no specific chromosomal marker comparable to the Ph¹ chromosome has been found in PV, studies have shown that up to 25% of untreated patients may demonstrate chromosomal abnormalities in the marrow at time of diagnosis (Westin et al, 1976; Wurster-Hill et al, 1976; Testa, 1980). The most common cytogenetic findings are hyperdiploidy, particularly trisomy 8,9,12, and 19. A more specific cytogenetic defect, in about 20% of patients is the 20q11 deletion which is rarely found in other hematological disorders (Millard et al, 1968; Westin et al, 1976; Zech et al, 1976). Structural rearrangements such as deletions and translocations, aneuploidy, polyploidy and hypoploidy are also found (Kay et al, 1966; Westin et al, 1976; Wurster-Hill et al, 1976). Karyotypic aberrations are more extensive in patients with a longstanding history of the disease, and in those who have been treated with ³²P or alkylating agents (Testa, 1980).

The role of chromosomal changes in the etiology of PV is unknown. Although a higher incidence of karyotypic abnormalities is seen in treated PV patients when the disease transforms into acute leukemia (Testa, 1980), no direct correlation was seen between the presence of chromosomal abnormalities and progression to acute leukemia (Wurster-Hill and McIntyre, 1978). Conversely, the presence of a normal karyotype in PV does not eliminate the possibility that the disease will terminate in a leukemic transformation. The prognostic value of cytogenetic findings in PV at this time appears to be very limited.

Ep-independence The presence of an increased cell mass without a concomitant increase in Ep levels led early on to the speculation that erythropoiesis in PV patients was autonomous, i.e. outside the regulatory control of Ep (Adamson, 1968). However, a number of in vivo observations indicate that some degree of normal responsiveness to erythropoietic stimuli may be present in PV patients. For example, both phlebotomy and hypoxia lead to an increased production of Ep (Gurney, 1973), and concomitant with the appearance of Ep in the urine, an increase in iron turnover and reticulocyte production occurs, indicative of augmented erythropoiesis (Adamson, 1968).

In vitro studies utilizing the plasma clot (Prchal and Axelrad, 1974), agar (Horland et al, 1977) and methycellulose (Aye, 1977; Eaves and Eaves, 1978; Lacombe et al, 1980) have demonstrated the presence of erythroid progenitors in PV capable of colony formation in culture without the addition of Ep. Such "Ep-independent" erythroid colony formation is not seen in normal subjects, or in patients with secondary polycythemia if care is taken to ensure that levels of Ep in other components of the culture (e.g. in the fetal calf serum) are insignificant (Eaves et al, 1980). Controversy exists however, as to whether Ep-independent progenitors present in PV patients are truly autonomous to the normal requirement for continuing contact with Ep. From studies reporting the reversible inhibition of such colony formation by the addition of Ep and anti-Ep antiserum preparations (Weinberg, 1977; Zanjani et al, 1977) and the failure of Ep-independent erythroid colony growth in serum-free cultures (Casadevall et al, 1982), it has been suggested that erythroid progenitors in PV may be exquisitely sensitive to Ep and can satisfy their requirement from the trace amounts of the hormone contributed by the fetal calf serum in the culture medium. Other in vitro experiments have provided evidence that at least some erythroid precursors in PV retain a

normal Ep responsive mechanism. A stimulatory effect of added Ep on the number of colonies obtained in PV culture was first demonstrated by Prchal and Axelrad (1974) and has subsequently been confirmed by a number of investigators (Aye, 1977; Eaves and Eaves, 1978; Zanjani et al, 1979); Eaves et al, 1980). This suggests that in PV at least a proportion of erythroid progenitors may be subject to normal regulation by variable Ep levels in vivo. Ep dose-response studies have confirmed that two populations of erythroid progenitors exist in PV: 1) a phenotypically abnormal population capable of colony formation in culture containing less than .001 u/ml of Ep, and 2) a phenotypically normal population with a normal Ep responsiveness (Eaves and Eaves, 1978). The use of G6PD isoenzymes markers in female heterozygotes with PV have shown that all erythroid precursors capable of Ep independent colony formation are members of the abnormal clone (Prchal et al, 1978b). However, the ratio of isoenzyme types obtained from colonies cultured in the presence of optimal levels of Ep was indicative of a subpopulation of cells belonging to the abnormal clone but not capable of endogenous colony formation. Ep-independent growth can thus be considered a unique, but not consistent marker of the abnormal clone. This finding is supported by more recent experiments in which the distribution of Ep independent and Ep dependent colony forming cells among the progeny of single BFU-E was examined (Cashman et al, 1983). The data obtained from these experiments established that primitive BFU-E belonging to the neoplastic clone as shown by their ability to produce Ep-independent colony forming cells could also produce substantial numbers of Ep-dependent progenitors, and thus suggests that phenotypic expression of Ep-independence is not fixed prior to the BFU-E stage.

Ep-independence is not restricted to the MPD; cells from murine fetal liver (Johnson and Metcalf, 1977), neonatal lamb bone marrow (Roodman and Zanjani, 1979) and human cord blood (Tchernia et al, 1981) also variably express the potential for endogenous colony formation. We have suggested therefore that the capacity for Ep-independent growth associated with stem cell transformation may not be a "new" acquisition but may represent the reemergence of a fetal characteristic (Cashman et al, 1983).

The results of a recent survey of a large number of unselected patients with PV demonstrate that unlike CML or ET, Ep-independence is a constant feature of all clones that lead to this disease regardless of treatment or time from diagnosis (Eaves et al, in press). It would be expected that low circulating levels of Ep in the PV patient would provide a considerable growth advantage for abnormal cells that could complete the erythropoietic program under such conditions, hence permitting them to dominate the mature cell compartment. However, the demonstration that only one isoenzyme type is found in the circulation, even when red cell levels (and hence presumably Ep levels) are brought down to normal in the treated patient, is at variance with Ep-independence being the sole pathological mechanism (Prchal et al, 1978b). Furthermore such a phenotypic advantage does not account for the increase in granulopoiesis and megakaryopoiesis usually seen in these patients.

Cell culture studies In vitro studies of progenitors in PV have demonstrated a number of characteristics similar to those of CML cells, such as alterations in cell cycle activity (Fauser and Messner, 1981), elevated CSA levels (Metcalf, 1977), high proportions of buoyant light density colony-forming cells (Singer et al, 1980), and normal ratios of primitive erythroid to granulocytic progenitors, although in PV, unlike CML, the frequency of

these progenitors is not elevated (Eaves and Eaves, 1979). This latter finding would indicate that early commitment events are not altered in the MPD, but rather that the preferential amplification of the progeny of the abnormal clone occurs at later stages of maturation, perhaps by a mechanism that confers a growth advantage on clonal cells while suppressing the proliferation of normal progenitors.

Several lines of evidence support the hypothesis that such a mechanism may operate in PV. Unlike the situation seen in CML, in vitro colony assays using cells from G6PD heterozygotes indicate that substantial numbers of non-clonal progenitors are present in PV patients (Prchal et al, 1978b). Since direct analysis of the peripheral blood could not detect the progeny of these normal cells, even in patients with normal counts (Adamson et al, 1976), their further maturation was blocked in vivo. The proportion of erythroid progenitors which are not members of the abnormal clone decreases substantially as cells progress down the differentiation pathway (Fialkow et al, 1978; Eaves and Eaves, 1979). This predominance of abnormal progenitors in later compartments has led to the suggestion that normal erythropoiesis may be suppressed at a differentiation step between BFU-E and CFU-E (Adamson et al, 1980). Analysis of the G6PD isoenzyme type of individual colonies before and after exposure to ^3H -thymidine demonstrated that a porportion of clonal CFU-C were in S-phase, while non-clonal CFU-C were quiescent (Singer et al, 1980). Since in normal marrow up to 40% of CFU-C are killed by exposure to ^3H -thymidine (Metcalf et al, 1974), these data would suggest that in PV the proliferation of normal granulocytic progenitors may be suppressed.

Alternations in the cell cycle regulation of hemopoietic progenitors in PV have been found by some authors, but not confirmed by others. No differences were noted in the ratio of normal and neoplastic BFU-E from G6PD

heterozygotes after exposure to ^3H -thymidine (Singer et al, 1979c). Similar results were obtained by other investigators using Ep-independence as a marker for the abnormal clone (Mladenovic and Adamson, 1982). These findings contradict the observations of Fauser and Messner (1981) who found an increased fraction of circulating Ep-dependent and -independent BFU-E and CFU-GEMM in active cell cycle, and of Zanjani and his associates (1978) who demonstrated an increased sensitivity to ^3H -thymidine suicide of endogenous CFU-E. The possible role of increased cell cycle activity in the numerical expansion of the abnormal clone in PV therefore remains obscure.

In summary, PV is a disease of clonal origin, that may be described as a neoplastic disorder, in which the progeny of a single stem cell, insensitive to normal growth regulatory mechanisms and perhaps with a proliferative advantage, completely fill the mature cell compartments in vivo. Unlike CML residual normal stem cells have been readily demonstrable in vitro. However, as the disease progresses these may decrease in number (Adamson et al, 1980). Although chromosomal abnormalities are found in a minority of patients with PV, there is no direct evidence yet as to which genetic rearrangements might play a primary role in the origin and evolution of the neoplastic characteristics of the abnormal clone.

C) Essential Thrombocytosis (ET)

ET (primary, hemorrhagic or idiopathic thrombocythemia) is characterized by abnormal proliferation of the megakaryocytes resulting in increased platelet production. The most common clinical manifestations are hemorrhage and/or thrombosis. It occurs most frequently in the fifth or sixth decade of life, equally among males and females, though a second peak of onset occurs in younger adults with a strong female preponderance (Silverstein, 1985).

ET may be distinguished from PV or CML by a platelet count in excess of $10^6/\text{mm}^3$, with marked marrow megakaryocytic hyperplasia and absence of the Ph¹ chromosome. The red cell mass is usually normal, with adequate iron stores if previous bleeding episodes have been kept under control. A modest leukocytosis is a common finding. Defects in platelet function have been reported with abnormalities in aggregation and adhesiveness, but platelet survival is normal. Hemorrhagic or thrombotic complications may become life threatening when the platelet count rises above $10^6/\text{mm}^3$, and may be lowered with myelosuppressive drugs, ³²P, or thrombocytapheresis (Wintrobe, 1976).

ET was firmly established as a MPD when its clonal origin in a pluripotent stem cell was demonstrated (Fialkow et al, 1981). Clinical remission in ET is not accompanied by the emergence of nonclonal progenitors. A minority of patients undergo transition to another MPD, including PV, CML, or myelofibrosis, or may evolve into an acute leukemia (Silverstein, 1985).

Karyotypic abnormalities are rare in ET. Only 5% out of 170 of ET analyzed during one study were considered to have a definite chromosomal abnormality (Third International Workshop on Chromosomes in Leukemia, 1981). No common anomaly was noted.

In vitro studies in ET have demonstrated a number of similar stem cell characteristics with other MPD. Endogenous erythroid colony forming cells have been described in ET (Prchal and Axelrad, 1974; Wong and Tobin, 1979; Partenen et al, 1983; Eridani et al, 1983; Eaves et al, 1983b) and like the other MPD, addition of Ep results in an increase in the number and size of the erythroid colonies formed. An elevation in the number of circulating CFU-M has been found in ET (Hoffman et al, 1983; Hibben et al, 1984). The presence of endogenous megakaryocytic colonies (i.e. colonies formed independent of the addition of conditioned media) has been documented in MPD

with thrombocytosis suggesting that abnormalities in responsiveness to pathway specific regulators may be a common pathological mechanism in the MPD (Gerwitz et al, 1983).

5) THESIS OBJECTIVES

This series of experiments was undertaken to elucidate the role of altered cell kinetics in the pathogenesis of the MPD. Though numerous authors have studied the proliferative characteristics of CFU-C in CML, comparable data for the erythroid lineage was not available at the time this study was initiated. More extensive studies had been attempted in PV with the examination of the cycling characteristics of erythroid and granulocytic progenitors but these had yielded contradictory results in different laboratories. At the present time an extensive review of the literature indicates that the cycling characteristics of hemopoietic progenitors in ET still have not been studied elsewhere.

Since the possibility exists that the contradictory results obtained by various other groups were due to differences in culture conditions and progenitor cell classification, the need was obvious for a complete examination of the cell cycle characteristics of all classes of hemopoietic progenitors in each of the MPD by a single laboratory under identical conditions.

Accordingly, progenitor cell cycle studies were undertaken on marrow and peripheral blood cells from patients with CML, ET, and PV and from normal controls. The results of these studies, which are detailed in Chapter III of this thesis, demonstrated consistent differences in the proliferative activity of primitive, normally quiescent hemopoietic progenitors in patients from all MPD categories when compared to normal controls. These interesting

observations encouraged an extension of cell cycling studies in an in vitro system that might be amenable to manipulation and in which the basis of the cell cycle control of normal and neoplastic cells might be characterized and analyzed.

For a number of years investigators in our laboratory have been able to maintain primitive clonogenic hemopoietic progenitors in the adherent layer of normal long term marrow cultures for periods of up to 8-12 weeks (Coulombel et al, 1983). Application of the ^3H -thymidine cell suicide assay to normal long-term marrow cultures demonstrated a regulated proliferation of the most primitive hemopoietic progenitor types in this layer (see Chapter IV). It was of interest, therefore, to examine the cycling characteristics of progenitors from long term cultures established with cells from patients with MPD in order to determine if the abnormalities in proliferative behaviour seen in vivo could be reproduced in vitro.

Preliminary experiments had indicated that the long-term culture system could support the proliferation of marrow cells from PV patients. Such cultures were therefore established and evidence of persisting neoplastic cells sought. The cycling behaviour of primitive progenitors was then measured (Chapter V). On the other hand, previous application of long-term marrow cultures to the study of hemopoietic progenitors in CML had shown that with most CML patients, Ph^1 positive cells could not be maintained under these conditions. Recently, a modification of the long term culture system, using CML peripheral blood added to pre-established normal marrow adherent layers proved effective in maintaining Ph^1 positive progenitors in vitro for an extended period of time (Eaves et al, 1983). Experiments were therefore designed to look for changes in the cycling characteristics of all classes of clonogenic erythroid and granulopoietic progenitors present in normal long

term cultures as well as in control cultures initiated with normal blood progenitors on pre-established feeders. These then served as a basis of comparison for studies of neoplastic cells maintained under similar conditions (Chapter VI).

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C H A P T E R I I
M A T E R I A L S A N D M E T H O D S

1) Marrow and Peripheral Blood Preparation

Peripheral blood and marrow specimens were obtained with informed consent and collected in preservative free sterile heparin present at a final concentration of 50 U/ml and 100-400 U/ml respectively. Marrow buffy coat cells were separated from the majority of red cells using a two step procedure of light centrifugation followed by sedimentation at unit gravity for 10-20 minutes. The buffy coat rich plasma was then removed, washed twice in serum free Iscoves medium and resuspended in the same for plating.

Peripheral blood was separated by layering a 10 ml aliquot over 15 ml of 1.077 gm/ml Ficoll-Hypaque (LSM-Bionetics, Kensington, Maryland) and spinning at 800g for 30 minutes. The light density mononuclear cell fraction at the plasma/Ficoll-Hypaque interface was then carefully removed and washed twice in 10 ml of serum free Iscove's medium and diluted to an appropriate concentration for plating.

2) Hemopoietic Colony Assays

Erythropoietic colony forming units (CFU-E) and burst forming units (BFU-E) as well as granulopoietic (CFU-C) and pluripotent (CFU-G/E) progenitors were assayed in a previously standardized culture medium consisting of 0.8% methylcellulose in Iscove's medium, supplemented with 1% deionized bovine serum albumin (BSA), 30% fetal calf serum (FCS), 200 mM L-glutamine, and 10^{-4} M 2-mercaptoethanol (Eaves and Eaves, 1978; Eaves et al, 1984). In addition, 5 U/dish human urinary erythropoietin (purified in this

laboratory to a specific activity of >100 U/mg) (Krystal et al, 1984) and an appropriate concentration of phytohemagglutinin or agar stimulated human leukocyte conditioned medium (Coulombel et al, 1983) was added to achieve optimal growth by all primitive clonogenic cell types (Gregory and Eaves, 1978). A 1.1 ml aliquot of this methylcellulose assay mixture, containing an appropriate concentration of hemopoietic cells was placed in each 35 mm Greiner Petri dish.

Routinely, normal marrow buffy coat cells were plated at a concentration of 2×10^5 cells per 1.1 ml culture. In normal peripheral blood assays, 4×10^5 cells were plated per 1.1 ml culture. However, if the patient's initial WBC count was elevated, additional dishes were set up at a lower concentration to ensure accurate colony counts. Adherent cells from long term bone marrow or peripheral blood cultures were routinely plated at a concentration of 1×10^5 cells per 1.1 ml culture. Nonadherent cells were also plated at this concentration, except in older cultures when the total number of nonadherent cells was insufficient and necessitated plating fewer cells.

Cultures were incubated at 37°C in a strictly controlled 5% CO_2 in air environment under conditions of high humidity. Each dish was scored on two different occasions. After 10 to 12 days of incubation small isolated clusters or pairs of clusters composed of 8 or more hemoglobin-containing cells were counted to determine the number of CFU-E plated. At this time, small bursts consisting of from 3 to 8 clusters of hemoglobinized cells were also scored to give mature BFU-E numbers. After an incubation period of 18-20 days all larger bursts, i.e. those containing 9 to 16 clusters, and those composed of more than 16 clusters as well as mixed colonies (CFU-G/E) were counted. In assays of fresh marrow and peripheral blood cells described in Chapter III, the term primitive BFU-E refers to high proliferative potential erythroid progenitors defined by their capacity to produce colonies containing

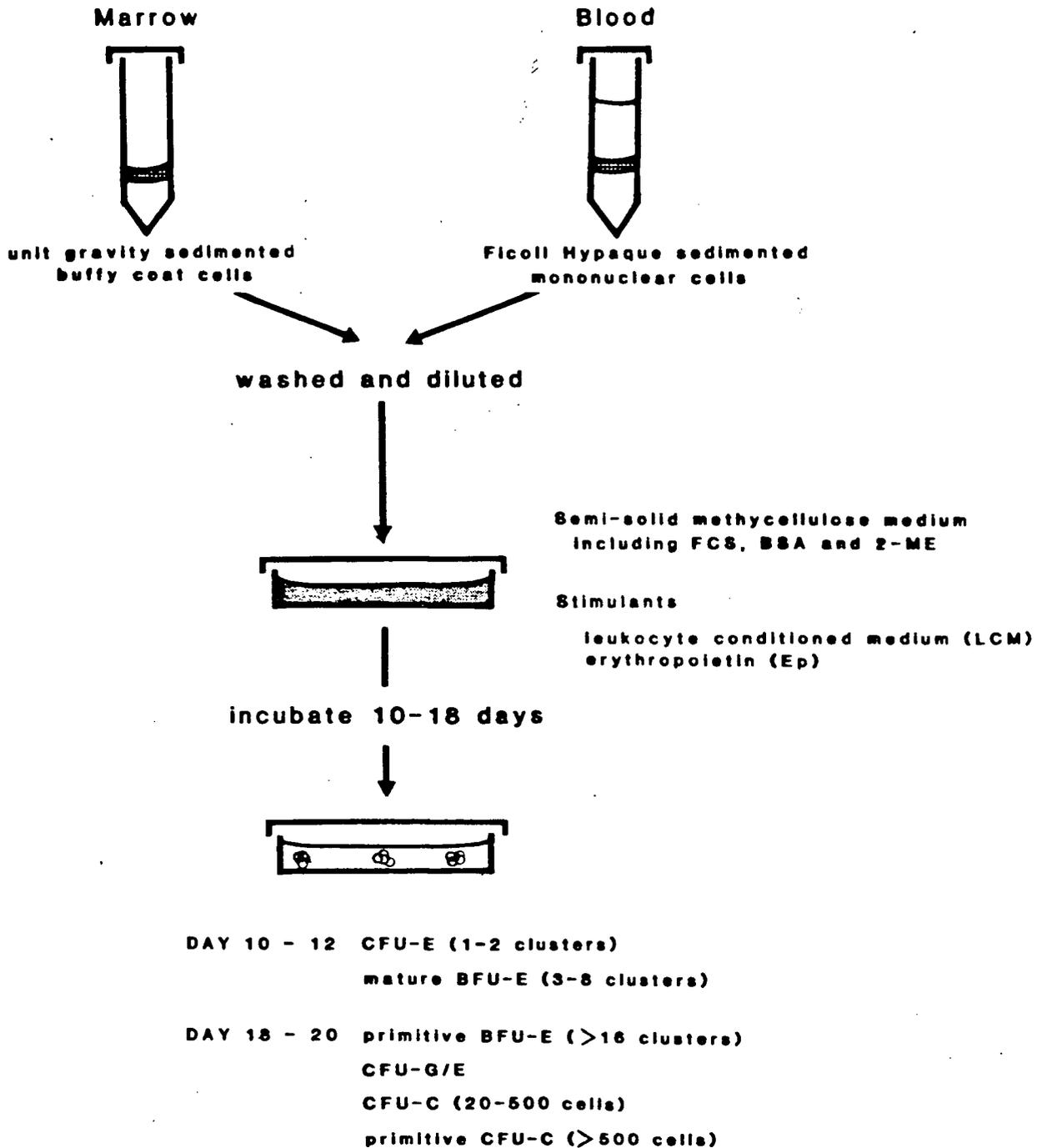


Figure 13. Schematic diagram describing the methylcellulose assay system for the growth of hemopoietic cells.

16 or more clusters. However, in the data presented in Chapters IV, V, and VI, the colony counts for all larger erythroid bursts were pooled, both for assays of nonadherent and adherent cells from long-term cultures as well as in assays of the fresh marrow and blood samples used to initiate these cultures. Thus, in these studies the term primitive BFU-E refers to clonogenic precursors capable of forming erythroid colonies containing 8 or more clusters. All granulocytic colonies were also scored at 18-20 days. These colonies were usually also subdivided into two categories, those containing 20-500 cells and those containing more than 500 cells. Values for the latter yield counts for primitive CFU-C numbers.

For assessment of progenitor numbers in primary or cultured cell suspensions, counts were averaged from 2-4 replicate 1.1 ml methylcellulose assay cultures. For assessment of cycling status counts were averaged from 6 replicates per treatment group.

3) Long Term Bone Marrow Cultures

An aliquot of the untreated marrow aspirate specimen containing $2-2.5 \times 10^7$ nucleated marrow cells was placed in 8 ml of growth medium in a 60x15mm Falcon tissue culture dish. The growth medium was composed of α medium supplemented with inositol (40 mg/l), folic acid (10 mg/l), extra glutamine (400 mg/l), fetal calf serum (12.5%), horse serum (12.5%), 2-mercaptoethanol ($10^{-4}M$), and hydrocortisone sodium succinate ($10^{-6}M$). The cultures were incubated for 3 to 4 days at 37°C in an atmosphere of 5% CO₂ in air. After this initial period of incubation all nonadherent cells were removed and layered over 1.077 gm/cc Ficoll-Hypaque to remove the red blood cells and mature granulocytes. The light density cells were washed in α medium supplemented with 2% FCS and returned to their original dishes

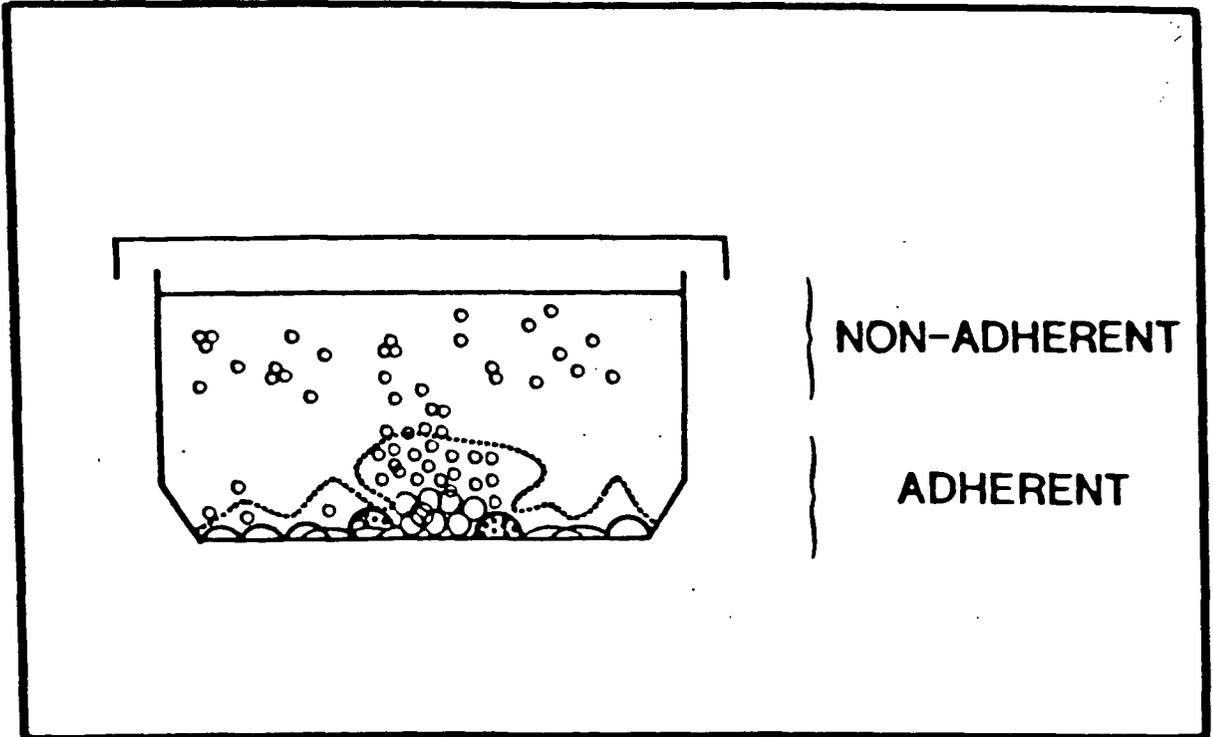


Figure 14. Schematic representation of the 3-layer composition of a long-term culture after 2-3 weeks of incubation.

From Eaves et al, 1983. Used with permission.

The cultures were fed on a weekly basis by removal of half of the medium and half of the nonadherent cells. This was accomplished by pipetting 2-3 ml of medium from the dish, and then gently swirling the dish to ensure removal of all the nonadherent cells with the remaining 6-5 ml of the medium. The culture medium was placed in a tube, vortexed to distribute the cells evenly, and 4 ml of this suspension was returned to the culture dish, along with 4 ml of fresh culture medium. The remaining 4 ml of growth medium containing half of the nonadherent cells was centrifuged, the growth medium removed, and the cells washed once in 2% FCS, counted, and assayed in methylcellulose cultures. If more than one culture was initiated from a single marrow specimen, the nonadherent cell fractions were pooled before washing.

4) Long Term Peripheral Blood Cultures

Marrow adherent layer "feeders" were obtained by subculturing primary 2 to 3 week old confluent normal marrow adherent layers established in the growth medium described above, but without the addition of hydrocortisone and fed weekly with removal of all nonadherent cells and complete replacement of the growth medium. The primary marrow adherent layer was suspended by treatment with trypsin (see procedure below), washed and counted. Aliquots containing $.5-1 \times 10^6$ cells were then placed in secondary culture dishes, and incubated at 37°C for 7 to 14 days until a confluent adherent layer was re-established. Just prior to use, these secondary adherent layers were irradiated with 15-20 gray (^{60}Co γ -rays or 280 kVp X-rays at a dose rate of 240 centigrays/min) to completely eliminate residual hemopoiesis.

Peripheral blood samples from CML patients were separated over Ficoll-Hypaque by centrifugation at 800 g for 30 minutes to obtain the mononuclear cell fraction. An aliquot containing $2.0-2.5 \times 10^7$ cells was then

added to the culture dishes containing the irradiated preestablished feeders in 8 ml of growth medium supplemented with hydrocortisone. These "reconstituted" blood cultures were subsequently handled as standard long-term marrow cultures.

Since normal blood samples contained fewer WBC per ml, the establishment of control cultures necessitated the processing of a large quantity of blood. This lengthy, multistep procedure for the separation of normal blood was performed by Dr. Louis Gaboury. A unit of normal blood, containing approximately 500 ml, was aliquoted into 50 ml tubes and centrifuged at 2000 rpm for 5 minutes. The buffy coat cells were then removed, and 10 ml aliquots of this cell suspension were then layered over Ficoll-Hypaque. After centrifugation at 800 g for 30 minutes the mononuclear cell fraction was removed and 100 U/ml of heparin was added to the cell preparation (to prevent subsequent clotting) which was then washed twice in 2% FCS and counted. The cell suspension was then diluted to a concentration of 5×10^6 cells/ml and subjected to the following T cell depletion procedure. A 1% suspension of 5-2-aminoethylisothiuronium bromide hydrobromide (AET) treated sheep red blood cells in 40% FCS was then added in equal volume to the cells. The tubes were incubated at 37°C for 5 minutes, centrifuged for 5 minutes at 150 g and then incubated at 4°C for 1 hour. After this period the supernatant from each tube was removed and the tubes centrifuged at 300 g for 10 minutes, the pellets resuspended and pooled for a second separation over Ficoll-Hypaque. The number of light density mononuclear cells recovered from the plasma/Ficoll-Hypaque interface after this step was approximately 3% of the original nucleated cell content and usually yielded sufficient cells for initiation of 2-3 long term cultures. These were subsequently maintained using the same protocol established for long-term marrow cultures and for reconstituted CML blood cultures.

In most experiments, with both CML patients and normal subjects, additional long term cultures were set up without pre-established feeders. These were initiated and handled in the same manner as the previous cultures except for designated dishes where all the nonadherent cells were returned to the cultures at each weekly half medium change.

5) Enzymatic Detachment of Adherent Cells

The adherent layer of long term marrow or peripheral blood cells was removed enzymatically using either collagenase (bacterial type I, 200 U/mg of protein) or trypsin (.25% in solution containing 5% citrate, 10% KCl and 1% glucose) following the procedure of Coloumbel et al (1983). The cultures were prepared for either procedure by removal of all the growth medium and nonadherent cells followed by a vigorous washing of the adherent layer with calcium- and magnesium-free Hank's balanced salt solution (HBSS-Ca-Mg). The additional detached cells were added to the nonadherent cell suspension.

The collagenase solution was prepared just prior to use by dissolving the collagenase in HBSS-Ca-Mg to a final concentration of 0.13% and sterilizing by passage through a 22 μ Millipore filter. To detach the adherent cells, 8 ml of the collagenase solution was pipetted onto the adherent layer, 2 ml of FCS was added, and the culture dishes incubated undisturbed for 3 hours at 37°C in an atmosphere of 5% CO₂ in air. When the cultures were removed from the incubator at the end of this period many cells were detached, and most of the remaining cells could be removed by gentle pipetting and washing. The cells were centrifuged at 300 g for 10 minutes to remove the collagenase solution and then washed twice in serum free Iscove's medium, carefully resuspended, counted, and diluted.

When trypsin was used, the cultures were prepared as for the collagenase procedure. Five ml of the enzyme solution was then placed in each of the culture dishes which were then incubated for 10 minutes at 37°C. At the end of this incubation period, 1 ml of FCS was added to stop further trypsin action, and all adherent cells could then be easily detached by gentle pipetting. Cells were then processed in the same manner as those harvested using the collagenase procedure.

6) The ^3H -thymidine Cell Suicide Assay

Each hemopoietic cell suspension was washed twice in serum free Iscove's medium prior to the ^3H -thymidine cell suicide assay to eliminate contamination by endogenous unlabelled thymidine. The cell suspension was resuspended in the same pre-warmed medium (pH 7.2) and the cells incubated with (tube T_T) or without (tube T_C) 20 $\mu\text{Ci/ml}$ ^3H -thymidine (25 Ci/mol) for 20 minutes at 37°C at a final cell concentration of 4×10^6 (blood), 2×10^6 (marrow), or 1×10^6 (adherent or nonadherent cells from long-term cultures) in a volume of 1 ml. Ten ml of cold thymidine (400 $\mu\text{g/ml}$) in 2% FCS was then added and the cells washed twice in the same medium prior to resuspension in 2% FCS in Iscove's medium and plating in methylcellulose. Suicide (or % kill) values were calculated from colony counts according to the formula in Figure 15. Values less than one are shown in the Tables as zero.

The concentration of ^3H -thymidine used in these experiments was selected on the basis of reported studies (Becker et al, 1965; Iscove, 1977) and confirmation of these results for the types of samples and reagents used in this study (see Chapter III). These showed that percent suicide values were the same whether cells were exposed to 20 or 100 $\mu\text{Ci/ml}$. In addition, in order to establish that there was no nonspecific toxicity due to incubation in

the ^3H -thymidine solution, other aliquots of the same marrow suspension were exposed to each ^3H -thymidine concentration in the presence of excess cold thymidine (400 μgms). This procedure was followed upon receipt of each new vial of ^3H -thymidine used in these experiments.

7) Cytogenetic Methods

Cytogenetic procedures were used in some of the studies of CML cultures to determine the presence or absence of the Ph^1 chromosome. Direct marrow metaphases were prepared by conventional methods (Tijo and Whang, 1962) using solid Giemsa staining or G banding (Seabright, 1971). In addition, metaphases were obtained from individual colonies from methylcellulose assays established with aliquots of the initial marrow specimen, or with the adherent or nonadherent cell fraction of long term cultures. This procedure was developed in the Terry Fox laboratory (Dube et al, 1981). Previous experience has shown that analyzable metaphases could be obtained by a careful selection of recognizable but immature colonies in which the largest number of dividing cells are present. To obtain such colonies, dishes were examined using an inverted microscope after 8 or 9 days incubation and subsequently at daily intervals. When a culture was judged to be optimal, 0.1 ml of colcemid (1 $\mu\text{g/ml}$ in HBSS) was carefully applied drop-wise over the methylcellulose surface of each 1.1 ml culture to arrest cell division at metaphase. A finely drawn out Pasteur pipette was used to remove individual single colonies which were each then transferred into a microtiter well containing 0.1 ml of .075M KCl. The cells in each colony were gently dispersed by pipetting and were left undisturbed in the microtiter wells for 15 to 20 minutes at 20°C . At the end of this time the entire contents of the microtiter well were transferred onto a microscope slide that had been coated 1-2 hours previously with a drop

of a 0.01% polylysine (w/v) solution. To spread the polylysine a coverslip was placed over the drop of polylysine until the slide was to be used. The coverslip was then removed, the slide rinsed with water, and gently blotted dry. Each colony was placed on the polylysine treated area of a single slide and then kept in a humid environment for 10 minutes. Excess hypotonic solution was removed with a cotton swab, and 0.5 ml of 3:1 methanol:acetic acid fixative gently dropped over the colony, followed by another 0.5 ml of the same fixative 30 seconds later. After 1 minute the slide was slowly air dried by passage over an open flame, then immersed in fresh fixative for 15 minutes, and air dried prior to staining.

All cytogenetic preparations were made by an experienced cytogenetic technician, and the analyses overseen by Dr. D.K. Kalousek.

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C H A P T E R I I I

ANALYSIS OF THE PROLIFERATIVE ACTIVITY OF HEMOPOIETIC PROGENITORS
IN THE MYELOPROLIFERATIVE DISORDERS1) INTRODUCTION

The myeloproliferative disorders - PV, ET, and CML - are a group of closely related syndromes characterized by a clinically significant increase in the number of circulating mature blood cells. Considerable cytogenetic and isoenzyme evidence has been presented for the pluripotent stem cell origin of all of these diseases (Nowell and Hungerford, 1960; Fialkow et al, 1967, 1977, 1981; Adamson et al, 1976; Jacobson et al, 1978). A number of investigators have applied in vitro colony assay techniques to the study of the MPD in an effort to elucidate the mechanisms that permit the progeny of a single abnormal stem cell to completely dominate all of the mature myeloid cell compartments. In CML, assessment of the number of clonogenic precursors in marrow and blood samples has provided evidence that these compartments are also greatly expanded. In PV and ET, where the increase in numbers of mature blood cells in any lineage is much less pronounced (usually less than a 2-3 fold increase) the size of the clonogenic progenitor compartments also remains on average within normal limits (Gregory and Eaves, 1977). Comparison of the relative numbers of clonogenic progenitors committed to different pathways of differentiation has revealed no evidence for a significant shift in the commitment behaviour of neoplastic pluripotent stem cells in any of these disorders (Eaves and Eaves, 1979; Adamson et al, 1980).

Demonstration of the clonal nature of all of these diseases, already apparent at the level of early progenitor cell types, focussed our interest on the assessment of progenitor cell cycle status as a possible mechanism contributing to clonal expansion of the circulating cell compartment in either the presence (CML) or absence (PV and ET) of a concomittant detectable increase in progenitor cell numbers. In view of the conflicting and incomplete cycling data reported by others for PV and CML progenitors (see Chapter I) and the lack of any published cycling data for ET progenitors, a systematic analysis of this parameter was undertaken. In these experiments primitive and mature erythropoietic, granulopoietic, and, in some cases, pluripotent progenitors, from both marrow and blood were assessed. To evaluate the specificity of cycling changes measured in the MPD, samples from patients with secondary erythrocytosis and various other secondary perturbations of hemopoiesis were also evaluated. Since some of the marrow and blood samples did not reach the laboratory for processing until several hours after being obtained, additional control studies included an evaluation of the effect of storage on ice and at room temperature on progenitor number and cycling status, in addition to standard ^3H -thymidine dose response measurements.

2) RESULTS

A) Patients

PV Sixteen PV patients were used in this study, ranging in age from 38 to 77 years. Cells from thirteen of the 16 patients were cultured at the time of diagnosis. In this group of untreated patients hemoglobin values ranged from 16.9-19.4 g/dl (5 women) and from 18.9-21.2 g/dl (8 men). Two other patients, both women, had been diagnosed 10 years earlier and had been

treated intermittently with hydroxyurea or myleran. Their hemoglobin values were 13.8 g/dl and 18/2 g/dl at the time of culture. One patient had been treated regularly by phlebotomy since the initial diagnosis had been made 5 years previously. Her hemoglobin was 15.3 g/dl at the time of culture. All PV patients studied had WBC values within normal limits or slightly elevated (range 7,500-16,300 cu/mm) and all demonstrated the presence of Ep-independent erythroid colony-forming cells in their peripheral blood and marrow (Eaves and Eaves, 1983)

SE Sixteen SE patients (13 men and 3 women) were used in this study. These ranged in age from 34-84 years. Cells for culture were in all cases obtained at the time of diagnosis. Hemoglobin values ranged from 16.4-20.1 g/dl (men) and from 15.7-18.8 g/dl (women). All SE patients had WBC within or close to normal limits (4,200-11,000 cu/mm). No Ep-independent erythroid colony-forming cells were detected in assays of blood and marrow from any of these patients.

ET Eight female and 11 male (i.e. a total of 19) patients with thrombocytosis were used in this study. These ranged in age from 32-83 years. Cells from 17 of these were cultured at the time of presentation. One patient, diagnosed a year previously had been treated with anturan. Another had been diagnosed 6 months previously, but had not required treatment. The platelet counts for all of the patients ranged from 912,000-2,632,000 cu/mm at time of culture. The WBC levels were normal or slightly elevated (range of 4,900-17,200 cu/mm). The hemoglobin values were normal (8.6-16.5 g/dl). Eight of 19 patients in this group had Ep-independent erythroid colony-forming cells present in their blood or marrow.

CML All 20 CML patients (12 females and 8 males, ranging in age from 24-82 years) in this study were Ph¹-positive. Four of these patients had

been previously treated and in this group WBC values ranged from 16,000-151,000 cu/mm. WBC values in the group of 16 remaining untreated patients ranged from 10,700-209,000 cu/mm. With the exception of the 4 treated patients, all members of this latter group were cultured within a year of diagnosis.

Normals Normal peripheral blood was obtained from laboratory personnel and from the Red Cross. Normal bone marrow specimens were obtained from 3 allogenic bone marrow transplant donors and from 17 patients undergoing hematological assessment for a variety of disorders including lymphoma, anemia, leukocytosis, and ankylosing spondylitis but whose marrow showed no malignant infiltration or other abnormality. The hematological indices for these individuals were normal, with the exception of the two male subjects with anemia, who had hemoglobin values of 8.2 and 8.0 g/dl.

Other patients Three other patients with perturbed hemopoiesis were included in this study. The first was a 26 year old female patient with a leukocytosis of unknown origin. She had a WBC count of 15,300 cu/mm, with a normal hemoglobin (12.1 g/dl) and platelet count (474,000 cu/mm). The second patient in this group was a 12 year old boy with thalassemia major. He had a WBC of 4,100 cu/mm, a platelet count of 134,000 cu/mm and a hemoglobin of 9.1 g/dl. The third patient was a 59 year old male with thrombocytopenia. He had a platelet count of 30,000 cu/mm, a WBC of 4,200 cu/mm and a hemoglobin of 15.5 g/l. None of these 3 patients demonstrated the presence of Ep-independent erythroid colony-forming cells in their blood or marrow.

B) ³H-thymidine Dose Response Curve

Percent suicide values for erythroid and granulocytic progenitors from 6 normal marrows exposed to 0, 10, 20, and 100 μ Ci/ml of ³H-thymidine are shown in Figure 15. The results for actively cycling hemopoietic progenitors (i.e. CFU-E, CFU-C, and all BFU-E) indicated that ³H-thymidine

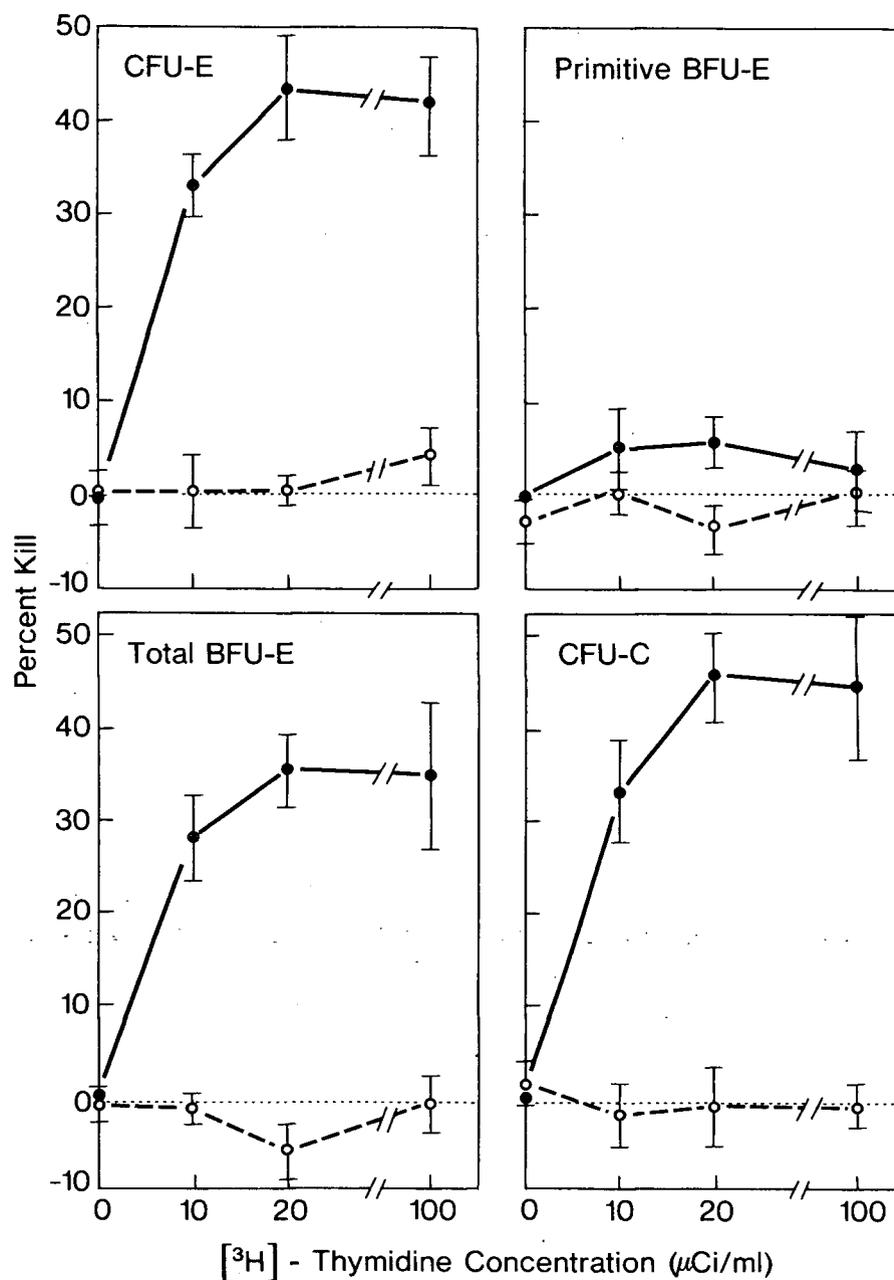


Figure 16. ^3H -thymidine dose response curve for erythroid and granulocytic progenitors from normal marrow. Each point represents the arithmetic mean \pm 1 SEM of values obtained from 6 experiments. The solid line (closed symbols) indicates values for cells incubated for 20 minutes in tubes containing only the appropriate concentration of ^3H -thymidine in a nucleoside-free nutrient medium. The dotted line (open symbols) refers to the values obtained when aliquots of the same cell suspension were incubated at each ^3H -thymidine concentration plus an excess (400 $\mu\text{g/ml}$) of cold thymidine.

concentrations of 20 μ Ci/ml gave maximum percent kill values with no change when the ^3H -thymidine concentration was increased up to 100 $\mu\text{Ci/ml}$. In contrast, the plating efficiency of primitive BFU-E (i.e. erythroid progenitors forming colonies consisting of more than 16 clusters) in the same marrow cell suspensions was not affected by exposure to any of the concentrations of ^3H -thymidine tested.

The absence of toxicity in the ^3H -thymidine solution, unrelated to its ^3H content, was also demonstrated in this series of experiments. In the presence of excess cold thymidine the percent kill at each concentration was reduced to insignificant levels indicating that cell death resulting from incubation in the ^3H -thymidine solution in each case was attributable to the specific incorporation of the radioisotope into S-phase cells.

C) Cell Cycle Status of Hemopoietic Progenitors From Marrow and Blood

Differences in progenitor cell cycling behaviour between normal subjects and PV or CML patients were most readily apparent in the circulating cell compartments (Table 1). In normal subjects and patients with SE all classes of circulating hemopoietic precursors were found to be quiescent. In contrast, in patients with PV or CML, a significant elevation in the number of S-phase progenitors was readily evident. A similar alteration in the cycling behaviour of PV or CML marrow could also be seen at the level of the most primitive progenitor compartment of the erythroid and granulocytic lineages. In later compartments, most progenitors were found to be in S-phase even in normal subjects (Table 2).

The results obtained for peripheral blood and marrow progenitors from a group of patients with elevated platelet counts are summarized in Tables 3 and 4. The alterations in cell cycle activity observed with the blood and marrow progenitors from CML and PV patients was not a consistent finding

TABLE 1

PERCENT KILL FOR PERIPHERAL BLOOD PROGENITORS FROM NORMAL INDIVIDUALS
AND FROM PATIENTS WITH MYELOPROLIFERATIVE DISORDERS

| Diagnosis | No. of Subjects | BFU-E (>2 clusters) | CFU-C | CFU-GEMM |
|-----------|--------------------|------------------------|------------|------------|
| Normal | 9 | 0 ± 1.2 | 0 ± 1.9 | 0 ± 2.0 |
| SE | 12 | 0 ± 1.0 | 1.25 ± 1.8 | 0 ± 3.5 |
| PV | 14 | 33.2 ± 2.7 | 26.4 ± 3.4 | 46.7 ± 4.9 |
| CML | 16 | 34.1 ± 3.3 | 32.2 ± 3.9 | 48.0 ± 5.1 |

Values shown are the arithmetic means ± 1 S.E.M.

TABLE 2

PERCENT KILL FOR MARROW PROGENITORS FROM NORMAL INDIVIDUALS
AND PATIENTS WITH MYELOPROLIFERATIVE DISORDERS

| Diagnosis | No. of Subjects | Primitive BFU-E (>16 clusters) | Primitive CFU-C (>500 cells) | CFU-GEMM |
|-----------|-----------------|--------------------------------|------------------------------|----------|
| Normal | 20 | 3.6 \pm 1.7 | 4.4 \pm 2.0 | - |
| SE | 15 | 2.5 \pm 2.2 | 4.9 \pm 2.9 | 0 |
| PV | 16 | 46.8 \pm 3.4 | 40.4 \pm 3.8 | - |
| CML | 13 | 40.4 \pm 3.6 | 44.2 \pm 7.0 | - |

Values shown are the arithmetic mean \pm 1 S.E.M.

TABLE 3
 PERCENT KILL FOR PERIPHERAL BLOOD PROGENITORS
 FROM PATIENTS WITH ESSENTIAL THROMBOCYTOSIS

| Group ¹ | Patient No. | Ep Independence ² | BFU-E (>2 clusters) | CFU-C | CFU-GEMM |
|--------------------|-------------|------------------------------|---------------------|-------|----------|
| I | 1 | No | 0 | 0 | 0 |
| | 2 | No | 0 | 0 | —* |
| | 3 | No | 3 | 5 | — |
| | 4 | No | 3 | 0 | — |
| | 5 | No | 0 | 3 | 0 |
| | 6 | No | 0 | 0 | 0 |
| | 7 | No | 1 | 5 | 0 |
| | 8 | No | 0 | 0 | — |
| | 9 | No | 0 | 9 | — |
| | 10 | No | 0 | 0 | 0 |
| | 11 | No | 2 | 10 | 11 |
| II | 1 | Yes | 30 | 30 | — |
| | 2 | Yes | 67 | 23 | — |
| | 3 | Yes | 22 | 12 | — |
| | 4 | Yes | 32 | 29 | — |
| | 5 | No | 43 | 22 | — |
| | 6 | Yes | 52 | 46 | — |
| | 7 | Yes | 25 | 29 | — |
| | 8 | Yes | 52 | 60 | — |

¹ - Patients were divided into these two groups on the basis of the proliferative activity of their circulating progenitor cells. No clinical correlation was evident within each group.

² - Ep-independence was determined by the presence of detectable erythroid colonies in culture dishes to which no Ep was added (<.002 U Ep/ml).

* - Insufficient counts for meaningful % kill calculations.

TABLE 4
 PERCENT KILL FOR MARROW PROGENITORS
 FROM PATIENTS WITH ESSENTIAL THROMBOCYTOSIS

| Group ¹ | Patient No. | Ep Independence ² | Primitive BFU-E (>16 clusters) | Primitive CFU-C (>500 cells) | CFU-GEMM |
|--------------------|-------------|------------------------------|--------------------------------|------------------------------|----------|
| I | 1 | No | 39 | 0 | —* |
| | 2 | No | 10 | 8 | — |
| | 3 | No | 0 | 10 | — |
| | 4 | No | 5 | — | — |
| | 5 | No | 0 | 6 | 0 |
| | 6 | No | 0 | — | — |
| | 7 | No | 6 | 0 | — |
| | 8 | No | 0 | 8 | — |
| | 9 | No | 11 | 6 | — |
| | 10 | No | 18 | — | — |
| II | 1 | Yes | 75 | 8 | — |
| | 2 | Yes | 47 | 16 | — |
| | 3 | Yes | 28 | 22 | — |
| | 4 | No | 53 | — | — |
| | 5 | Yes | 46 | 41 | 39 |
| | 9 | Yes | 36 | 15 | — |

¹ - Patients were divided into these two groups on the basis of the proliferative activity of their circulating progenitor cells. No clinical correlation was evident within each group.

² - Ep independence was determined by the presence of detectable erythroid colonies in culture dishes to which no Ep was added (<.002 U Ep/ml).

³ - Insufficient counts for meaningful % kill calculations.

for all patients studied. However, when these latter patients were divided into two groups on the basis of the proliferative activity of their circulating progenitor cells, it became apparent that many patients did not show an increase over normal values in the number of progenitors in S-phase in their peripheral blood or marrow. In addition, alterations in cycling behaviour, when present, were usually found in both the marrow and peripheral blood compartments alike. The only marked exception to this was patient no. 1 where cycling was apparent in the primitive BFU-E compartment in the marrow but not in the peripheral blood. As seen with the other MPD patients, such cycling abnormalities were not lineage specific, i.e., both erythroid and granulocytic progenitors were affected, regardless of whether the patient was diagnosed as having PV, CML, or ET.

Another interesting association was revealed when erythroid progenitor cells from these patients were assessed for their ability to form recognizable, hemoglobin-containing colonies without the addition of Ep. The presence of such endogenous erythroid colony forming cells has previously been documented in ET patients (Eaves et al, 1983; Eridani et al, 1983; Partanum et al, 1983). As shown in Tables 3 and 4, in 7 out of 8 experiments with peripheral blood cells, and 5 out of 6 experiments with marrow cells, an increase in the number of hemopoietic progenitors in S-phase was found only in those patients in whom endogenous erythroid colony forming cells were also detected.

In contrast to the MPD the proliferative activity of all classes of hemopoietic progenitors from the bone marrow or peripheral blood of 3 patients with various other forms of perturbed hemopoiesis (i.e. reactive leukocytosis, thalassemia major or thrombocytopenia) were indistinguishable from normal values.

TABLE 5
THE EFFECT OF VARYING TEMPERATURE AND TIME OF SPECIMEN STORAGE ON THE PERCENT KILL OF HEMOPOIETIC PROGENITORS

| Experiment | Progenitor Class | DIAGNOSIS | | | | | | | | | | | | |
|--------------------|---------------------|-------------------|-------------------|------------------|-------------------|-------------------|-------------------|-------------------|--------------------|------|-------|---------|----|----|
| | | NORMAL | | | ET | | | CML | | | PV | | | |
| | | Fresh | 8 hours | | Fresh | 8 hours | | Fresh | 8 hours | | Fresh | 8 hours | | |
| | 0°C | 22°C | 0°C | 22°C | 0°C | 22°C | 0°C | 22°C | 0°C | 22°C | 0°C | 22°C | | |
| Blood - 1 | BFU-E | 0 | 3 | 4 | 0 | 0 | 1 | 35 | 38 | 44 | 48 | 49 | 44 | |
| | | 2 | 0 | 0 | 4 | 52 | 46 | 47 | 26 | 34 | 27 | 44 | 45 | 42 |
| | | 3 | 0 | 2 | 4 | | | | | | | | | |
| | late* CFU-C* | 1 | 1 | 0 | 1 | 0 | 0 | 12 | 22 | 25 | 32 | 37 | 32 | 41 |
| | | 2 | 0 | 3 | 0 | 46 | 52 | 45 | 47 | 49 | 36 | 37 | 49 | 49 |
| | | 3 | 5 | 0 | 0 | | | | | | | | | |
| early** CFU-C** | 1 | - | - | - | 0 | 0 | 0 | 40 | 26 | 28 | 49 | 42 | 48 | |
| | 2 | 0 | 9 | 5 | 43 | 54 | 46 | 50 | 55 | 60 | 52 | 63 | 57 | |
| | 3 | 0 | 0 | 0 | | | | | | | | | | |
| CFU-GEMM | 1 | - | - | - | 0 | - | 13 | - | - | - | 54 | 45 | 56 | |
| | 2 | - | - | - | - | - | - | 31 | 40 | 56 | 54 | 39 | 62 | |
| Marrow - 1 | early BFU-E* | 0 | 5 | 0 | 11 | 13 | 6 | 60 | 50 | 50 | 50 | 42 | 59 | |
| | | 2 | 0 | 4 | 4 | 57 | 56 | 51 | 47 | 36 | 45 | 25 | 32 | 29 |
| | | 3 | 9 | 0 | 6 | | | | | | | | | |
| | mature** BFU-E** | 39 | 56 | 47 | 57 | 52 | 46 | 14 | 23 | 24 | 36 | 37 | 45 | |
| | | 37 | 35 | 33 | 46 | 55 | 58 | 45 | 46 | 33 | 36 | 47 | 37 | |
| | | 30 | 20 | 31 | | | | | | | | | | |
| | late CFU-C | 43 | 37 | 40 | 42 | 41 | 48 | 12 | 25 | 30 | 42 | 40 | 36 | |
| | | 45 | 46 | 45 | 59 | 46 | 56 | 51 | 51 | 47 | 14 | 18 | 13 | |
| | | 48 | 47 | 50 | | | | | | | | | | |
| | early CFU-C | - | - | - | 5 | 0 | 6 | - | - | - | 32 | 50 | 22 | |
| | | 6 | 0 | 0 | 48 | - | - | - | - | - | - | - | - | |
| | | - | - | - | 48 | - | - | | | | | | | |
| mean of difference | | 1.0 | .06 | .69 | 1.14 | .77 | 2.77 | 1.07 | 2.0 | | | | | |
| t value | | .52 ^{ns} | .05 ^{ns} | .4 ^{ns} | .56 ^{ns} | .34 ^{ns} | .84 ^{ns} | .43 ^{ns} | 1.26 ^{ns} | | | | | |
| degrees of freedom | | 17 | 17 | 12 | 13 | 12 | 12 | 14 | 14 | | | | | |

* : late CFU-C - 20-500 cells/colony; ** : early CFU-C - >500 cells/colony

+ : early BFU-E - >16 clusters/colony; ++ : mature BFU-E - 3-8 clusters/colony

^{ns} : not significant - no significant difference was seen in percent kill of hemopoietic progenitors between the fresh specimen aliquot processed immediately and the aliquots kept at 0°C and 22°C and processed 8 hours later as determined by the Students T test.

D) Time Course Experiments

A number of experiments were undertaken to examine the effect of time and temperature on the proliferative activity of hemopoietic progenitors from the blood and bone marrow of MPD patients and normal subjects. These studies were necessary to demonstrate that the values shown in Tables 1-4 were not affected by variations in the interval between removing the cells from the patient and testing the cells for their sensitivity to ^3H -thymidine. To evaluate the effect of storage time on ^3H -thymidine suicide values, freshly obtained MPD specimens were placed on ice or kept at room temperature for 8 hours before processing. In this series of experiments all normal and patient peripheral blood and bone marrow specimens were obtained locally and were processed within an hour of their removal from the subject to generate zero storage time values.

For periods of up to 8 hours there was no detectable effect of storage on the absolute numbers or cycling status of the hemopoietic progenitors in any given sample ($p > 0.5$) (Table 5). This was true whether the specimen was from a normal subject or a MPD patient. It would appear therefore, that the consistent differences observed here in the cell cycle activity of hemopoietic progenitors from patients with MPD as compared to normal subjects cannot be accounted for by differences in accrual procedures.

3) DISCUSSION

The present studies confirm those of others indicating an increase in progenitor turnover with progression down the erythroid or granulocytic differentiation pathway. Pluripotent progenitors, capable of mixed colony formation in vitro are quiescent in normal marrow (Fauser and Messner, 1979) as are the majority of the most primitive erythroid progenitors (Eaves et

al, 1979). The present studies show that the most primitive granulopoietic progenitors, as identified by their high proliferative capacity are quiescent also. More mature progenitor types characterized by a lower proliferative potential normally show a significant proportion of their numbers in S-phase. Such differentiation stage-specific alterations in proliferative activity have also been noted in the mouse (Gregory and Eaves, 1978). The absence of S-phase progenitors in the circulation of normal individuals also confirms previous findings in man (Ogawa et al, 1977; Tebbi et al, 1976).

The mechanisms that regulate the cycling status of progenitors in the marrow and peripheral blood are not known. Both negative signals leading to quiescence, and positive signals leading to activation may exist and some evidence for localized changes in such signals has been reported (Gidali and Lajtha, 1972; Lord, 1979). In this context the apparent abnormalities of cycling behaviour observed in the MPD might then be attributed to 1) an intrinsic block within the cells that prevents responsiveness to negative regulatory signals, and/or 2) the activation of a mechanism(s) of autostimulation within the cells that overrides an extrinsically derived negative signal.

An increase in the suicide index after exposure to ^3H -thymidine and hence an increase in the number of cells in S-phase, may not necessarily result in an expansion of intermediate compartments if the transit time through these is decreased. However the final result expected in both situations would be an increase in the number of terminally differentiating cells as is typical in the MPD.

The application of clonal assay systems to the study of the MPD has demonstrated other common growth and in vitro progenitor differentiation

characteristics, such as the presence of Ep-independent erythroid progenitor cells (Prchal and Axelrad, 1974; Eaves and Eaves, 1978, 1979; Lacombe, 1980; Eaves et al, 1983) and increased numbers of light density CFU-C (Moore et al, 1973; Greenberg et al, 1976; Metcalf, 1977; Singer et al, 1980). The findings presented in this study indicate that abnormalities in progenitor proliferative behaviour may also be a common feature of primitive cells in different MPD clones. However, such alterations do not readily account for the preferential expansion of one mature cell type characteristic of each of the MPD, since the progenitor cycling changes observed were not lineage specific. Thus other anomalies in growth characteristics, such as altered production or sensitivity to regulatory factors resulting in a growth advantage of a particular cell line may act in tandem with alterations in cell cycle activity to produce the disease state.

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C H A P T E R I V

REGULATED PROLIFERATION OF PRIMITIVE HEMOPOIETIC PROGENITOR CELLS
IN LONG-TERM HUMAN MARROW CULTURES1) INTRODUCTION

Under normal conditions very few of the most primitive hemopoietic progenitor cells in the marrow are actively cycling, although following various treatments this situation may be altered (Becker et al, 1965; Eaves et al, 1979; Fauser and Messner, 1979). Data from a number of studies suggest that changes in the cycling activity of primitive progenitors may be mediated at least in part by mechanisms that operate locally within the marrow and involve cell types that do not commonly circulate (McCulloch et al, 1965; Gidali and Lajtha, 1972). These findings have provided considerable impetus for developing an in vitro model where the same mechanism(s) that regulate primitive hemopoietic cell behavior in vivo continue to operate. A system of this type could then be used to characterize the relevant cell types and analyze their mode of action. Dexter and co-workers have obtained two lines of evidence that the long-term mouse marrow culture system may represent such a model. They have found first, that the maintenance of pluripotent stem cells (CFU-S) in such cultures is dependent on the presence of other cells whose function is adversely affected by the S1/S1^d genotype (Dexter and Moore, 1977) and second, that the cycling activity of the stem cell population maintained is regulated by feeding or simple physical disturbance of the culture milieu (Toksoz et al, 1980).

Recently conditions for supporting primitive human hemopoietic progenitors for periods of 8 weeks or more in what appear to be similar cultures have been described (Gartner and Kaplan, 1980; Greenberg et al, 1981; Coulombel et al, 1983). I therefore undertook to evaluate the cycling status of primitive progenitor cells in such cultures and to look for comparable changes in this parameter that might be related to changes in culture conditions subject to manipulation.

2) RESULTS

A) Patients

Fifteen marrow specimens were used in this study to establish a total of 82 long term cultures. The specimens were obtained from 2 normal bone marrow donors and from 13 individuals undergoing hematological assessment for a variety of disorders including lymphoma, anemia, leukocytosis, and ankylosing spondylitis but whose marrow showed no malignant infiltration or other abnormality.

B) Cellularity and composition of the adherent and nonadherent fractions

For each experiment the nonadherent fraction was assessed on a weekly basis for total cellularity and numbers of progenitors in standard methylcellulose cultures. Individual long term cultures from each experiment were sacrificed after varying periods of incubation and the adherent layer assessed in the same manner. The results are shown in Fig. 17.

As noted by other investigators (Coulombel et al, 1983, 1984), the total number of nucleated cells in the nonadherent fraction declined rapidly during the first four weeks of incubation, to an average value of 2×10^5 cells/dish. During the next several weeks there is a "plateau" phase where the nonadherent cell count per dish remained relatively constant despite weekly

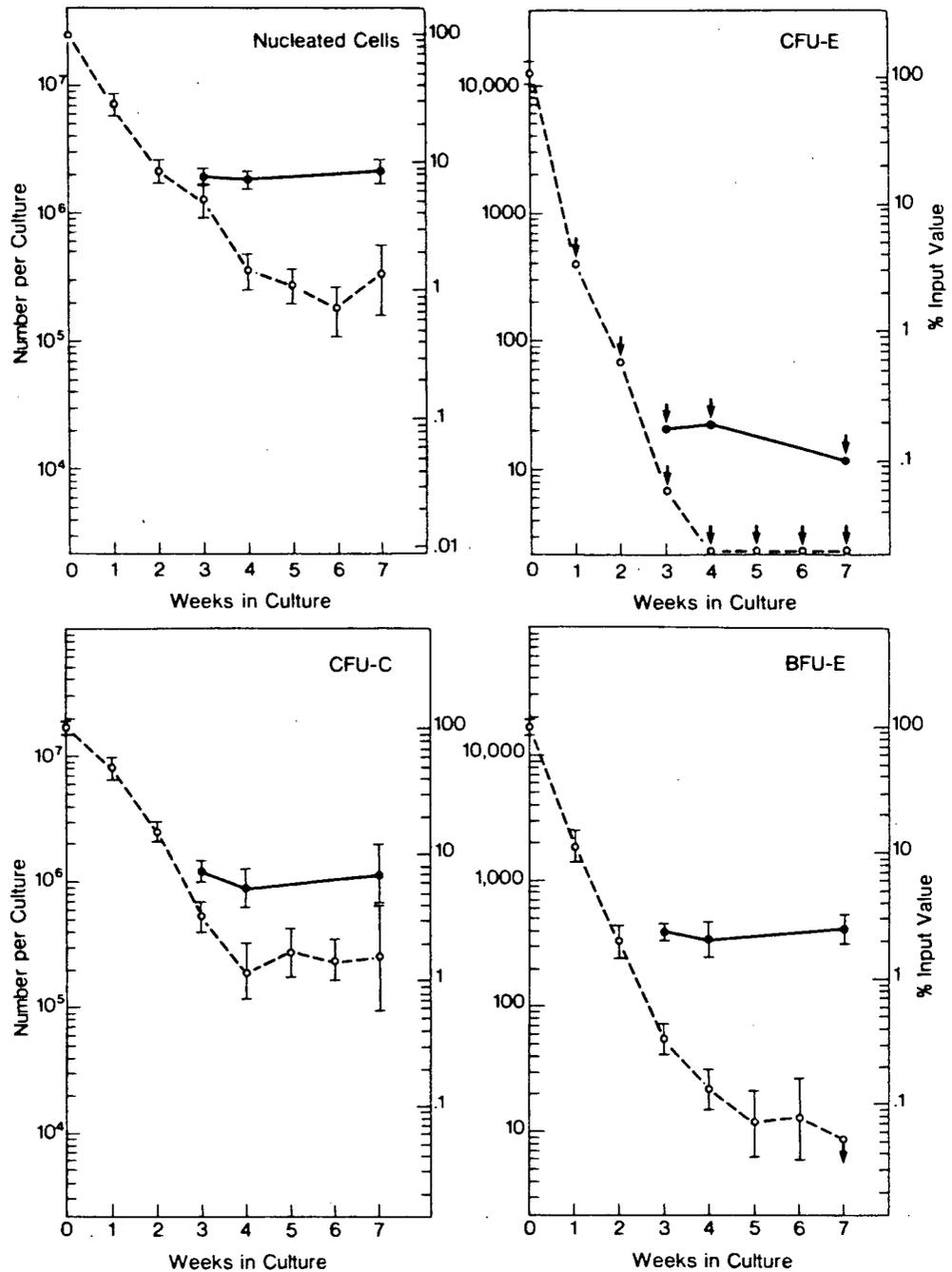


Figure 17. The cellularity and progenitor content of the adherent (solid line) and nonadherent (dotted line) fractions assessed at varying incubation times. Each point shown represents the geometric mean \pm SEM. Of the 15 experiments initiated, 5 were terminated at 3 weeks, 6 at 4 weeks, and 4 were maintained until 7 weeks and sacrificed for assessment at that time. The downward arrows indicate maximum mean values obtained if one colony had been seen in any of the assay dishes scored in each individual experiment.

removal of half of the nonadherent cells. In contrast the number of nucleated cells per adherent fraction of the same experiments demonstrated very little variation between 3 and 7 weeks.

The values obtained on a weekly basis from the nonadherent fraction for CFU-E, BFU-E, and CFU-C declined rapidly during the first four weeks of culture. Subsequently, progenitor values remained fairly constant or decreased more slowly. The most rapid decline in progenitor numbers over time was seen in the CFU-E compartment, and these could not usually be detected after two weeks. BFU-E were present in the nonadherent fraction for up to 7 weeks, but at low levels. The initial decrease in CFU-C numbers was not as pronounced as that seen in the erythroid compartment, and from 4 to 7 weeks of culture CFU-C values remained at a higher level than observed for either CFU-E or BFU-E.

Higher values for each progenitor class were obtained in the adherent fraction compared to the nonadherent fraction and these values remained relatively constant for up to 7 weeks.

C) ^3H -thymidine suicide assay

The effect of exposing cells to high specific activity ^3H -thymidine for 20 minutes was usually determined both for progenitors present in the original marrow used to initiate long-term cultures and for progenitors obtained from long-term marrow cultures after various periods of maintenance. Whenever possible nonadherent progenitors as well as those released by collagenase treatment of the adherent layer were evaluated. Total progenitor numbers in both fractions were similar to previously published values (Coulombel et al, 1984). As shown in Figure 18 and 19 assessment of primitive BFU-E and CFU-C starting populations showed these to include a low or undetectable S-phase component. Just prior to each weekly medium change,

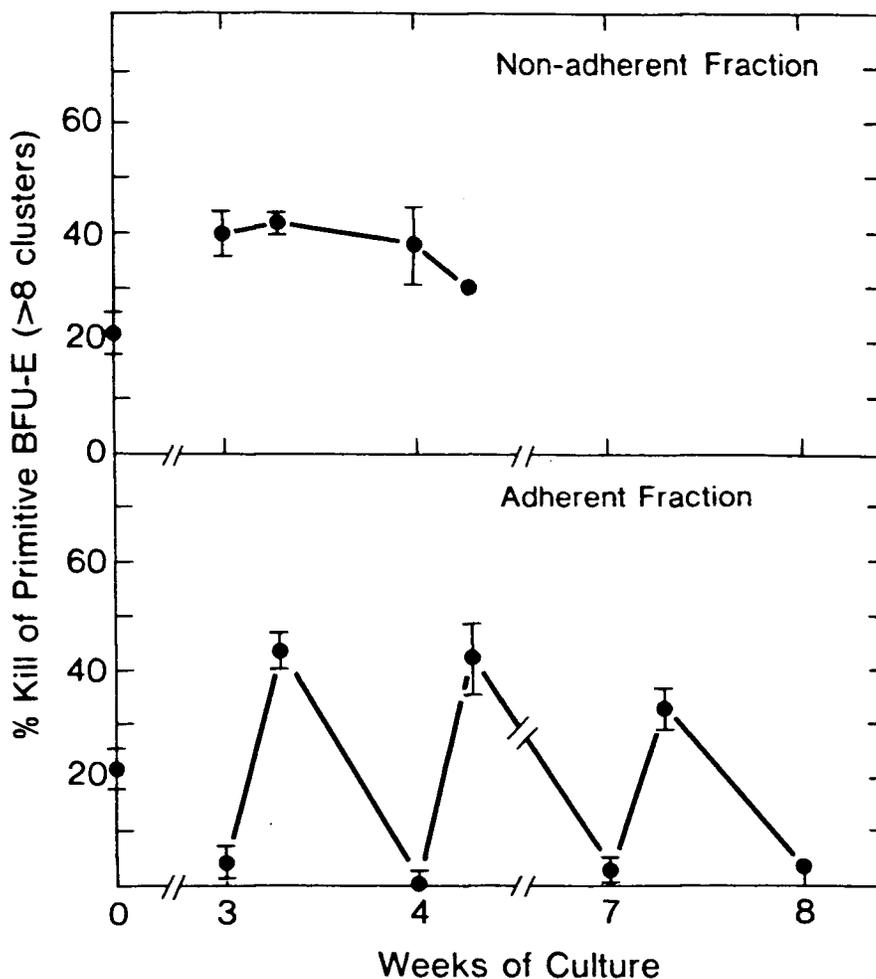


Figure 18. Thymidine suicide values for primitive BFU-E present in normal long-term marrow cultures assessed at various times after feeding. Cells were exposed to ^3H -thymidine immediately after removal from the cultures and then plated in methylcellulose as described in the text. Each value represents the arithmetic mean + 1 S.E.M. of values from 1 (no error bars) to 10 experiments (different marrows).

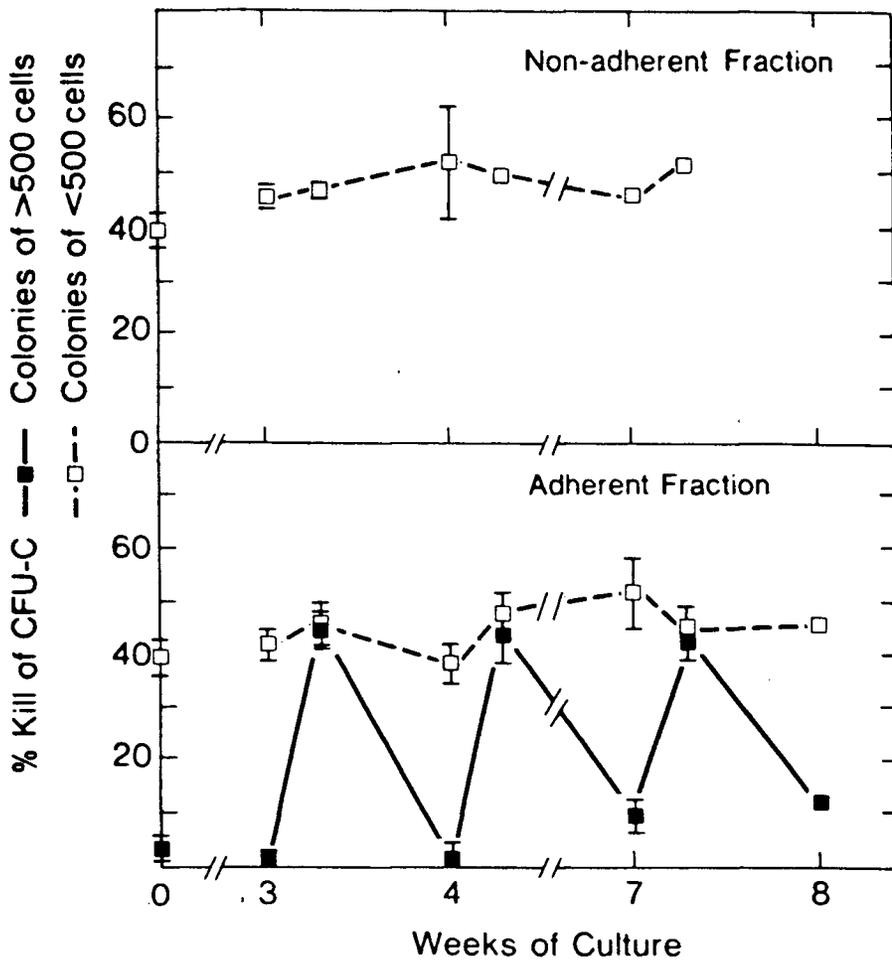


Figure 19. Thymidine suicide values for CFU-C present in normal long-term marrow cultures assessed at various times after feeding. Cells were exposed to ^3H -thymidine immediately after removal from the cultures and then plated in methylcellulose as described in the text. Each value represents the arithmetic mean + 1 S.E.M. of values from 1 (no error bars) to 10 experiments (different marrows).

a similar lack of detectable S-phase cells in the primitive BFU-E and CFU-C compartments of the adherent layer, where the great majority of these progenitor types are located, was routinely evident. Then 2 to 3 days later, the proportion of primitive BFU-E and CFU-C in S-phase regularly increased (Figs 18 and 19 lower panels). This pattern was observed in all 10 experiments undertaken (different marrows), irrespective of differences in the total number of primitive progenitors present or the age of the culture. Occasionally sufficient numbers of nonadherent primitive BFU-E were available for cycling determinations also. In contrast to primitive BFU-E in the adherent layer, those found in the nonadherent fraction were always actively cycling (Fig 18, top panel). High proliferative potential CFU-C were not found in the nonadherent fraction after 2 weeks as reported previously (Coulombel et al, 1983). However, large numbers of low proliferative potential CFU-C were maintained in both the nonadherent and adherent fractions. Like the nonadherent primitive BFU-E, low proliferative potential CFU-C in both fractions remained continuously in cycle (Fig 19). As expected, the numbers of low proliferative potential BFU-E detected were too small to permit meaningful assessment of their proliferative state.

Table 6 shows the results of experiments in which the effect of the physical manipulations involved in a routine medium change was tested independently. In these experiments the cultures were handled as before, except that all 8 ml of the old medium and all of the nonadherent cells were simply returned to the culture and no fresh medium was added. It can be seen that this was insufficient to activate the primitive progenitor cells present in the adherent layer into DNA synthesis. In some of these experiments the effect of removing half of the nonadherent cells (without changing the medium) or of adding fresh glutamine and fresh hydrocortisone (in the amount

Table 6. Effect of Different Feeding Procedures on Thymidine Suicide Values (% Kill) of Primitive Hemopoietic Progenitors in the Adherent Layer of Normal Long-Term Human Marrow Cultures*

| Expt. | Age of Culture When Fed (wks) | Standard Medium Change 2 days previously ⁺ | | Mock Medium Change 2 days previously [†] | | No Medium Change but Fresh Glut/HC Added 2 Days Previously [‡] | |
|-------|-------------------------------|---|--------|---|--------|---|--------|
| | | BFU-E* | CFU-C* | BFU-E* | CFU-C* | BFU-E* | CFU-C* |
| 1 | 3 | 59 | 60 | 0** | 8 | 0 | 0 |
| 2 | 3 | 44 | 55 | 0** | 0 | 4 | 0** |
| 3 | 3 | 56 | 36 | 4 | 5 | 7 | 11 |
| 4 | 4 | 46 | 36 | 6 | 9 | 16 | 16 |
| 5 | 7 | 35 | 46 | 0 | 0 | - | - |
| 6 | 7 | 39 | - | - | - | - | - |

*Values shown are for primitive BFU-E (>8 clusters) and primitive CFU-C (>500 cells). Negative values (**) ranging from -5 to -12% are shown as 0. Simultaneously recorded values for CFU-C (<500 cells) in the adherent layer ranged between 31% and 66% kill.

⁺Half of the medium replaced with fresh medium and half of the nonadherent cells removed.

[†]All of the medium and nonadherent cells were first removed as for a standard medium change, but both were then returned and no new medium was added.

[‡]1.2 mg of glutamine and 40 ul of 10^{-4} M hydrocortisone were added to cultures that were otherwise not handled.

normally added at each medium change) was also examined. Neither of these treatments was found to be sufficient to trigger primitive adherent layer progenitors into S-phase (Table 6).

3) DISCUSSION

The majority of clonogenic erythropoietic and granulopoietic progenitor cells present in human marrow are normally actively cycling although those characterized by a particularly high proliferative potential can be shown to represent a small, quiescent subset (Figs 18 and 19) (Eaves et al, 1979). In this respect they resemble other primitive hemopoietic progenitor cell types identified by their pluripotentiality (Fauser and Messner, 1979) or, in the mouse, a capacity for spleen colony formation in irradiated recipients (Becker et al, 1965). The implication of these findings is that common stage-specific (but not lineage specific) mechanisms may serve to control the proliferative behaviour of all of these early progenitor types.

The data presented here support such a model. They demonstrate that all of the various erythropoietic and granulopoietic progenitor populations detected in long-term human marrow cultures are proliferatively active, but when subclassified according to their demonstrated proliferative potential show marked differences in the regulation of their cycling status. Specifically, low proliferative potential progenitors were found to cycle continuously regardless of their location, whereas in the adherent layer high proliferative potential progenitors of both erythropoietic and granulopoietic lineages underwent periodic activation and re-entry into a non-cycling state. This unique behaviour of committed but high proliferative potential progenitors is similar to that previously documented for spleen colony-forming cells in murine long-term cultures (Tokoz et al, 1980). However,

although the mechanisms involved appear to be reproduced in human marrow cultures also, in this system they are restricted to progenitor types in the adherent layer. This difference between human and murine cultures may be due to the more cohesive structure of the adherent layer typical of the former and its greater tendency to retain the most primitive hemopoietic cells within its framework. A similar explanation may underlie the failure of physical perturbations associated with feeding of human long-term cultures to reproduce the activating effect of adding new growth medium.

How a balance between quiescence and activation of primitive hemopoietic cells is regulated is not yet resolved. Simple nutrient exhaustion seems unlikely to be a factor since low proliferative potential progenitors co-existing in the adherent layer or even primitive cells present in the non-adherent fraction proliferate continuously. A possible role of hydrocortisone or glutamine, two temperature-sensitive components of the medium has also been ruled out. Recent studies with peripheral blood progenitors cultured in the presence or absence of an irradiated marrow adherent layer show that progenitor proliferation occurs continuously in the absence of adherent marrow cells (see Chapter VI). In the presence of a feeder the pattern described for normal marrow cultures is reproduced (Eaves et al, 1985). Thus, a major part of the supportive function of cells in the adherent layer may lie not in an ability to activate primitive hemopoietic cells but in a function that forces primitive hemopoietic cells into a quiescent state and which itself can be regulated. Non-toxic, reversible negative regulators of primitive hemopoietic cells of murine origin have been described (Lord, 1979; Tokoz et al, 1980). The present studies suggest that analogous human factors exist and that they may be anticipated to act on early committed as well as pluripotent progenitors.

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C H A P T E R V

UNREGULATED PROLIFERATION OF PRIMITIVE HEMOPOIETIC PROGENITORS
IN LONG TERM POLYCYTHEMIA VERA MARROW CULTURES1) INTRODUCTION

Although several studies on the proliferative behaviour of hemopoietic progenitors in PV have now been reported, the possible role of increased cell cycle activity in the expansion of the abnormal clone in PV remains obscure. In Chapter III, results from a comprehensive study of progenitor cell cycle status in the MPD were presented and these clearly showed that alterations in primitive erythroid and granulocytic progenitor cell turnover are characteristic in these malignancies. Subsequent studies of normal progenitor populations maintained in the long-term marrow culture system showed that reproducible fluctuations between primitive progenitor quiescence and cycling could be observed, and it was further demonstrated that these changes were subject to modifications to the composition of the growth medium. The establishment of an in vitro model where normal primitive cells could thus be manipulated to enter and exit from the cell cycle provided an opportunity to evaluate its applicability for detecting abnormal components of this control mechanism in the MPD. Earlier work by another laboratory (Powell et al, 1982) had shown that standard long term cultures could be established with marrow from PV patients, and provided preliminary evidence that the neoplastic clone remained stable when maintained under these conditions. Initial experiments using Ep-independence as a marker for the abnormal clone confirmed that the culture conditions employed in our laboratory also did not result in selection for the normal clone in this disease, although the opposite finding had been documented for long-term cultures initiated with

CML marrow (Coulombel et al, 1983). Accordingly long-term cultures were established with marrow from a number of PV patients and cell cycle studies of the progenitors in both the adherent and nonadherent fractions of long-term cultures were preformed.

2) RESULTS

A) Patients

Cells from all 7 PV patients used in this study were cultured at time of diagnosis and therefore had not been previously treated. The hemoglobin values for the 3 women were 15.8, 16.7, and 20.6 g%. Corresponding values for the 4 men were 19.9, 20.0, 20.3, 20.5, and 20.6 g%. The patients ranged in age from 55 to 83 years. WBC values were within normal limits or slightly elevated (4,600-36,000 cu/mm). All patients demonstrated the presence of endogenous colony-forming cells in their blood and marrow (Eaves and Eaves, 1983). Data for many of the fresh specimens have also been included in the results reported in Chapter III.

B) Cellularity and Composition of PV Long Term Cultures

The total cellularity, as well as the CFU-E and CFU-C progenitor content of both the nonadherent and adherent cell fractions is shown in Figure 20. As in previous studies with normal cultures (see Chapter IV) the cellularity and composition of the nonadherent fraction in each experiment was determined on a weekly basis. For adherent layer assessment, individual long term cultures from each experiment were sacrificed 3,4, or 7 weeks after initiation.

The general behaviour of marrow cells from PV patients in long term culture was similar to that of marrow cells from normal individuals (see Chapter IV). An initial steep decline in total nucleated cell and progenitor

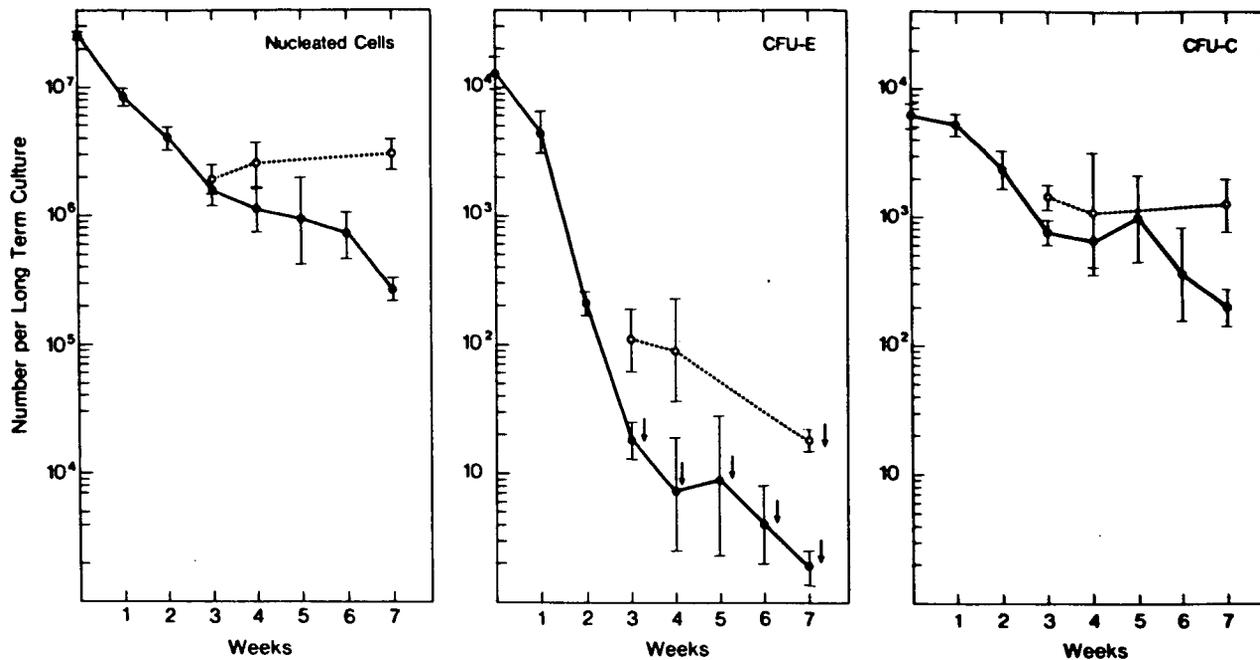


Figure 20. Total number of nucleated cells, erythroid colony forming cells and granulocyte progenitors in the adherent and nonadherent fractions of PV marrow cultures assayed at varying times after initiation. All cultures were established with marrow cells from newly diagnosed untreated patients. Each point represents the geometric mean \pm SEM. The downward arrows indicate maximum mean values obtained if one colony had been seen in any of the assay dishes scored in each individual experiment.

content of the nonadherent fraction occurred during the first 3-4 weeks of incubation. This was then followed by a "plateau phase" where these values remained fairly constant. The CFU-E compartment experienced the most rapid decline in progenitor number over time, with no, or very few CFU-E detectable in the nonadherent fraction after two weeks. The cellularity and composition of the adherent fraction in contrast to the nonadherent fraction, remained fairly constant throughout the duration of the experiment.

C) Erythropoietin-Independence in Long-Term PV Cultures

The presence of neoplastic erythroid progenitors capable of forming mature, hemoglobin-containing progeny in assay cultures that contained no added Ep was determined for both the adherent and nonadherent fractions of long term cultures. A similar assessment was also made on the initial marrow specimen used to establish the cultures. The results are shown in Figure 21. The proportion of erythroid progenitors from each fraction that was capable of Ep-independent colony formation remained constant regardless of the age of the culture, and was comparable to the values obtained in the marrow sample used to establish the culture. It would therefore appear that the abnormal clone remained stable in long term cultures established from these PV marrow aspirates.

D) ^3H -Thymidine Suicide Assay of Hemopoietic Progenitors

The proliferative activity of primitive and mature hemopoietic progenitors in the adherent fraction of 3, 4, and 7 week old cultures was determined. In the majority of experiments additional dishes were sacrificed 2 days later for similar progenitor cell cycling determinations. The results are shown in Table 7.

Regardless of the age of the culture, all classes of hemopoietic progenitors appeared to be in active cell cycle. This finding contrasts with

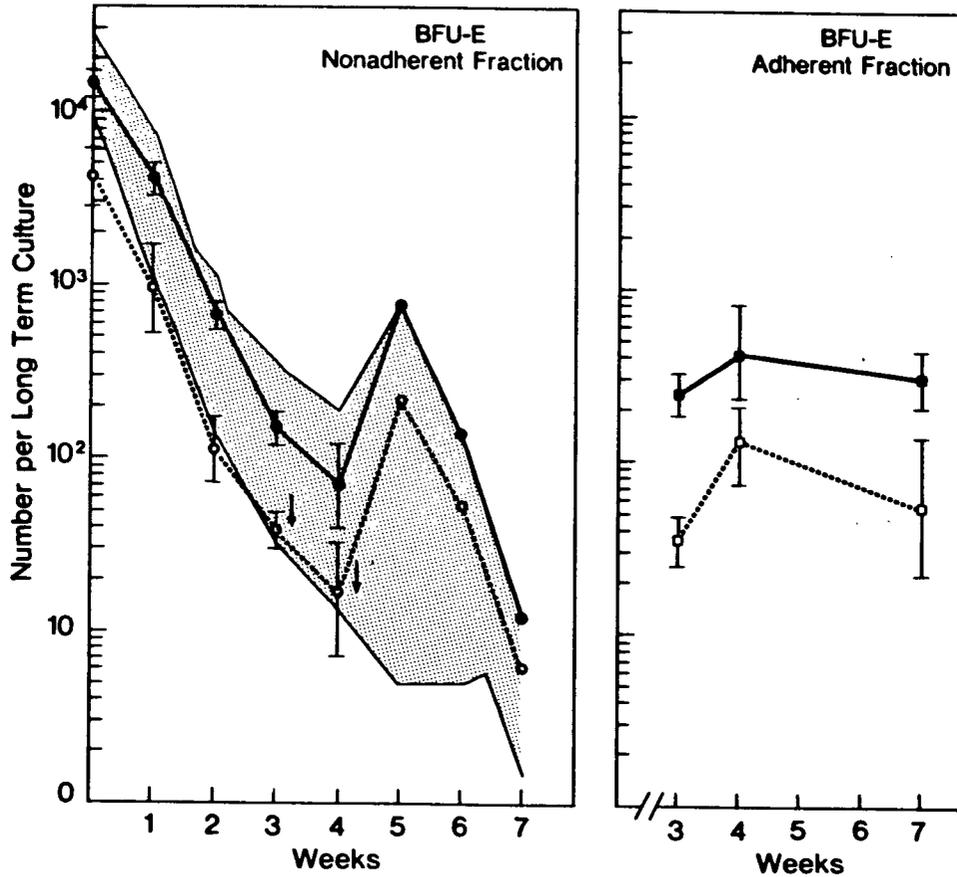


Figure 21. Assessment of BFU-E number in the adherent and nonadherent fractions of PV long term cultures at varying periods of incubation. Each point represents the geometric mean \pm SEM. The solid line indicates the total number of BFU-E present in cultures where the proportion of erythroid burst forming cells capable of colony formation in the absence of added Ep was also assessed (dashed line). Seven experiments were initiated of which 4 were terminated by week 4. Only 1 of the 3 experiments carried until week 7 produced sufficient numbers of nucleated cells to permit assessment of the proportion of endogenous colony forming cells. The solid region represents the range of nonadherent BFU-E number per long term culture for all 7 experiments, as determined by assay in methylcellulose cultures containing Ep. Similarly, the total number of BFU-E and the proportion of endogenous BFU-E was determined for the adherent layer. Cultures from 3 experiments were assayed in this manner at week 3, from 4 experiments at week 4, and from 3 experiments at week 7.

TABLE 7

THYMIDINE SUICIDE (% KILL) OF HEMOPOIETIC PROGENITORS
IN PV LONG TERM MARROW CULTURES

| Cell Fraction | Age of Culture | Exp. No. | BFU-E ¹ | | CFU-C ² | | |
|---------------|-----------------------|---------------------|--------------------|------|--------------------|------|----|
| | | | Early | Late | Early | Late | |
| Adherent | 3-4 weeks | 1 | 55 | 57 | - | 40 | |
| | | 2 | 32 | 47 | 30 | 61 | |
| | | 3 | 69 | - | 48 | 55 | |
| | | 4 | 50 | 54 | 53 | 54 | |
| | | 5 | 38 | 47 | 37 | 57 | |
| | | 6 | 58 | 47 | 57 | 55 | |
| | | 7 | 50 | 66 | 42 | 67 | |
| | 3-4 weeks + 2 days | 3 | 52 | 65 | 58 | 49 | |
| | | 4 | 42 | 71 | 53 | 46 | |
| | | 5 | 49 | 62 | 22 | 64 | |
| | | 6 | 62 | 50 | 54 | 52 | |
| | | 7 | 70 | 66 | 44 | 63 | |
| | 7 weeks | 5 | 43 | - | 11 | 57 | |
| | | 6 | 53 | - | 62 | 43 | |
| | | 7 | 53 | 64 | 48 | 51 | |
| | 7 weeks + 2 days | 7 | 51 | 34 | 66 | 59 | |
| | | | | | | | |
| | Non-adherent | 3-4 weeks | 3 | 47 | - | - | 64 |
| | | | 4 | 45 | - | - | 52 |
| | | | 7 | 52 | - | - | 47 |
| | | 4 weeks + 2 days | 7 | 56 | - | - | 62 |
| | | | | | | | |

¹ Early - >8 clusters; Late - 3-8 clusters

² Early - >500 cells; Late - 20-500 cells

the results obtained with normal cultures (see Chapter IV) where at week 3, 4, or 7, just prior to the weekly feeding, primitive BFU-E and CFU-C in the adherent layer were found to have a low or undetectable S-phase component. These normal early progenitors, however, could be triggered into active cell cycle by the weekly replacement of half of the culture medium. Such a pattern of regulated proliferation is not apparent in the cycling behaviour of primitive progenitors from long-term PV cultures. The majority of these cells were in S-phase irrespective of whether the cultures had been fed 7 days or 2 days previous to the ^3H -thymidine assay determinations.

In 3 experiments, a sufficient number of non-adherent cells were obtained to permit assessment of the proliferative state of progenitors in this fraction. As reported in Chapter IV for normal marrow cultures quiescent primitive BFU-E and late CFU-C were not found in this fraction of the culture at any time. Primitive CFU-C were not detected in the nonadherent fraction after 2 weeks, and mature BFU-E were found in insufficient quantity to permit meaningful determinations of their cell cycle status.

3) DISCUSSION

Studies on the proliferative behaviour of clonogenic progenitors in the marrow of normal individuals suggest that differentiation stage-specific mechanisms may regulate the cycling activity of primitive cell types. Subsequent studies suggested that conditions prevailing in the long-term marrow system may allow mechanisms to operate in vitro and hence be subjected to further analysis. In contrast, stage-specific alterations in progenitor proliferative activity are not observed in PV marrow where the majority of high proliferative potential progenitors of the erythroid and granulocytic

lineages appear to be actively turning over. In this series of experiments we have demonstrated that this unique pattern of continuous proliferative activity is also seen when PV cells are maintained in the long-term marrow culture system where, in normal controls, primitive cells become quiescent.

The mechanism that permits primitive PV progenitors to appear to bypass or ignore negative regulatory signals is not known. The defect could be intrinsic to the cell, such as an abnormality in the cell membrane of neoplastic cells that prevents normal interactions with negative regulatory cell types, or an abnormality in gene expression that leads to an autocrine phenotype. However, from the data presented here for PV, it is not possible to exclude faulty negative signal production as an explanation for the results reported in this Chapter.

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C H A P T E R VI

UNRESPONSIVENESS OF PRIMITIVE CML PROGENITORS
TO THE NEGATIVE REGULATORY EFFECT OF AN
ADHERENT CELL TYPE IN NORMAL MARROW1) INTRODUCTION

A minimal requirement for analyzing the molecular basis of the abnormal proliferative behaviour of primitive Ph¹-positive cells would be a culture system where they could be maintained and their turnover shown to be indifferent to normal control. As demonstrated in Chapter IV, normal primitive committed and pluripotent myeloid progenitors undergo cyclic changes in their proliferative behaviour in response to changes of the growth medium. However, the long-term system has not been useful for the study of CML progenitor regulation since primitive Ph¹-positive progenitor numbers rapidly decline when such cultures are initiated with CML marrow, even though conditions may be sufficient to support an initially undetectable but persisting normal progenitor population (Coulombel et al, 1983; Dube et al, 1984). In collaboration with others in the Terry Fox Laboratory, I therefore sought ways of modifying this type of culture system to achieve a higher yield of Ph¹-positive progenitors. Two approaches were evaluated. In the first, CML marrow cells were seeded onto pre-established adherent marrow "feeder" layers derived from normal long-term marrow cultures. In the second, CML peripheral blood (PBL) rather than marrow was used as a source of primitive Ph¹-positive progenitor cells and these were then seeded onto irradiated normal marrow adherent layers. As controls, allogeneic, T cell depleted, light density PBL cells from normal donors were also cultured on similar adherent marrow feeder layers. In each experiment progenitor numbers

in both the non-adherent and adherent fractions were determined after varying intervals by cloning in methylcellulose. Cycling characteristics of these cells were measured using the ^3H -thymidine suicide technique and progenitor genotypes in CML cultures determined by cytogenetic analysis of plucked colonies as described in Chapter II.

2) RESULTS

A) Patients

Marrow and/or PBL from 5 untreated CML patients were used for co-culture studies. Additional co-culture studies were performed with PBL from 2 other CML patients. These had been previously treated but had elevated WBC counts at the time of study and PBL progenitor numbers were greatly elevated. Marrow was also obtained from a previously studied patient (Dube et al, 1984) 6 years postdiagnosis and on hydroxyurea at the time the present aspirate was taken.

Normal PBL was obtained through the courtesy of the Canadian Red Cross. Normal marrow for the generation of adherent layers were from normal marrow transplant harvests or from lymphoma and Hodgkin's patients without marrow involvement.

B) Culture of CML marrow on normal marrow adherent layers.

The effect of seeding CML marrow aspirate specimens on irradiated normal marrow adherent layers established as described Chapter II was evaluated in 4 separate experiments using marrow cells from 4 different untreated CML patients. Results in all were similar. A representative experiment is shown in Figure 23. In general, the non-adherent fraction of cultures containing pre-established feeders was found to contain higher numbers of nucleated cells, BFU-E and CFU-C. Assessment of the adherent fraction required

sacrificing a whole culture and was therefore done less frequently (usually at 4 weeks and occasionally again at 7-8 weeks). Higher numbers of clonogenic progenitors were also found in the adherent fraction of CML marrow cultures established on feeders as compared to controls without feeders. Data for primitive progenitors is given in Table 8. Cytogenetic analysis of the large colonies produced from these progenitors showed that the maintenance of a significant population (>100 per culture) of primitive, i.e. high proliferative potential, Ph¹-positive progenitors had not been achieved in any experiment. Where larger numbers of primitive progenitors were present, these proved to be Ph¹-negative.

Thus, the use of feeders appeared to improve progenitor maintenance but only to a limited degree, and with little selectivity for the Ph¹-positive line. As a result, from none of these experiments were sufficient numbers of primitive Ph¹-positive progenitors obtained after 4 weeks to allow their cycling behaviour to be evaluated. However, in a previous study (Dube et al, 1981) we had identified an anomalous patient whose long-term marrow cultures maintained Ph¹-positive progenitor numbers in the range typical of cultures established from normal marrow. A marrow specimen obtained from this same patient 3 years later was used to initiate a second series of cultures. As shown in Table 9 extended maintenance of Ph¹-positive progenitors was again achieved. In anticipation that the numbers of those classified as primitive would be sufficient for cycling determinations, such measurements were also undertaken. As this proved to be the case these results are also presented in Table 9. It can be seen that primitive BFU-E and CFU-C in the adherent layer of these cultures did not become quiescent 7 days after a previous medium change as do their normal counterparts in control cultures (Chapter IV).

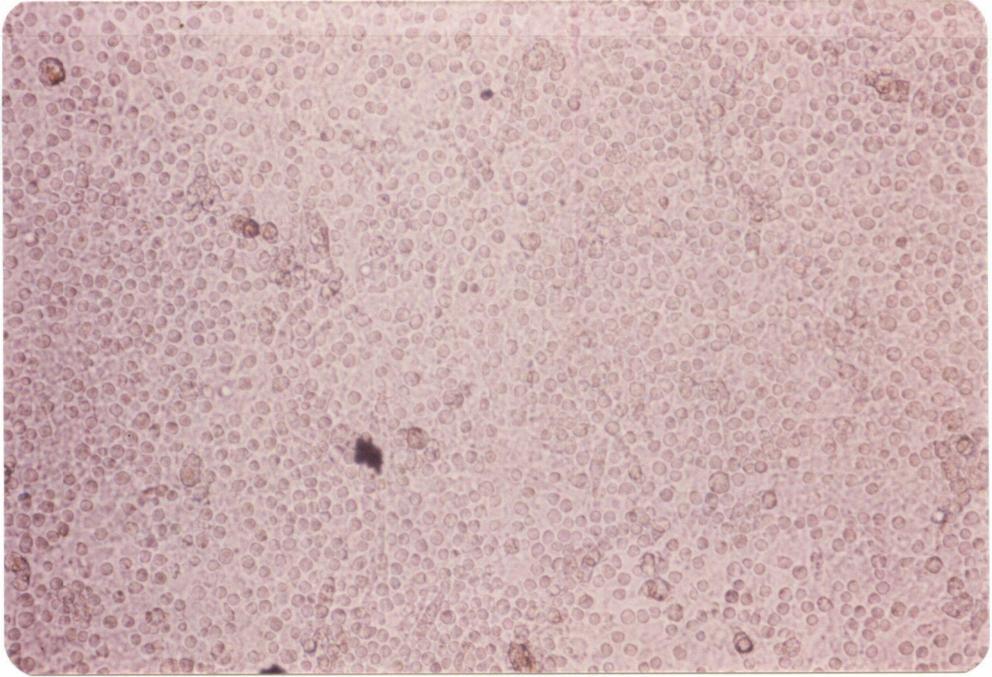


Figure 22. The adherent layer of a reconstituted CML long term blood culture 3 weeks after initiation (X160).

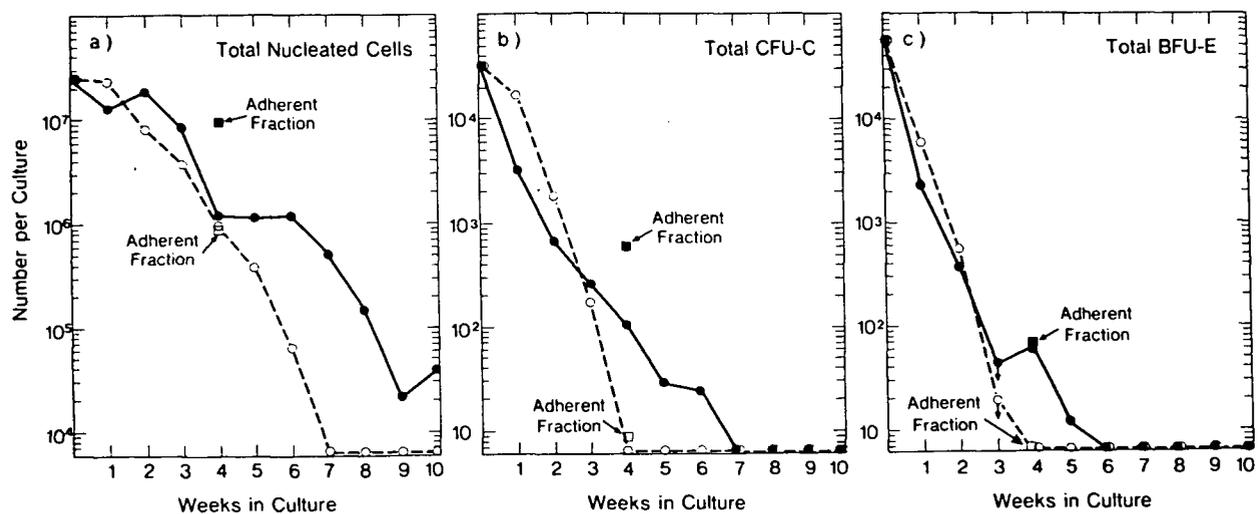


Figure 23. Comparison of total cell and progenitor content of a long-term CML marrow culture initiated with (solid symbols) or without (open symbols) a pre-established normal marrow feeder. Circles - data for the non-adherent fraction assessed weekly. Squares - data for the adherent layer assessed at week 4.

Table 8. Enhancing effect of feeders on primitive progenitor numbers maintained in long-term CML marrow cultures and assessed after 4 weeks

| Progenitor | Experiment no. | With feeders | | Without feeders | |
|----------------------------------|----------------|--------------|-------------------------------|-----------------|-------------------------------|
| | | No.* | Genotype (% Ph ¹) | No.* | Genotype (% Ph ¹) |
| Primitive BFU-E (>8 clusters) | 1 | 25 | 100 | 5 | -‡ |
| | 2 | 1320 | 0 | <26 | 0§ |
| | 3 | 52 | - | 3 | - |
| | 4 | 5 | 0 | 6 | 0 |
| Primitive CFU-C (>500 cells) | 1 | <13 | 100† | <5 | - |
| | 2 | 2260† | 0† | 1720† | 0† |
| | 3 | 26 | 100† | 3 | 100† |
| | 4 | 2 | 0 | 1 | 30† |

* Values shown are the number of primitive progenitors detected per 4 week culture from adherent layer assays. Except in 2 cases, where 11 and 6 primitive BFU-E per nonadherent fraction, respectively, were measured, no primitive BFU-E or CFU-C were detectable in this fraction. Values for total progenitor numbers were higher but similarly distributed.

† All types of CFU-C.

‡ No data.

§ From cytogenetic analysis of colonies derived from mature BFU-E.

Table 9. Primitive hemopoietic progenitor numbers and their cycling status at week 4 in long-term marrow cultures at week 4 (i.e. 7 days after the routine 3 week medium change) from an anomalous CML patient whose Ph¹-positive cells were maintained under these conditions.

| Progenitor* | Fraction assayed | Number | % Kill | Normal % kill values‡ |
|-----------------------------------|------------------|--------|--------|--------------------------|
| Primitive BFU-E (> 8 clusters) | Nonadherent | 946 | 46 | 38 \pm 7 |
| | Adherent | 280 | 35 | 0 \pm 3 |
| Primitive CFU-C (> 500 cells) | Nonadherent | 0 | -§ | - |
| | Adherent | 53 | 57 | 1 \pm 4 |

* All colonies analyzed at this time were found to be Ph¹-positive.

‡ % Kill values + 1 SEM from Chapter IV for these progenitor classes found in 4 week old long-term cultures established from normal marrows and handled in the same fashion.

§ No data.

C) Cultures of CML PBL on normal marrow adherent layers.

Since the PBL of untreated high count CML patients contains highly elevated numbers of neoplastic progenitors of all types, we next tested whether Ph¹-positive progenitor maintenance might be more reproducibly achieved when these were seeded onto pre-established irradiated normal marrow feeders. Preliminary experiments by my supervisor and others (Eaves et al, 1984) had revealed that such cultures rapidly assumed the appearance of hemopoietically active long-term marrow cultures and >1000 clonogenic progenitors per culture could commonly be detected after 4 weeks (Figure 22). This suggested that cycling studies of Ph¹-positive progenitors would be feasible using this type of reconstructed culture. Accordingly, a number of experiments were set up for this purpose. Figure 24 shows a summary of the progenitor maintenance achieved in the 11 experiments from 7 different CML patients. Assessment of their cycling status was also undertaken and the results of these studies are given in Table 10 and 11. From Figure 24 it can be seen that clonogenic progenitor numbers were typically maintained at levels in excess of 1000 per culture for periods of 4 weeks or longer when feeders were present, the majority being located in the adherent (feeder layer containing) fraction. In the absence of pre-established feeders, no adherent layer formed and virtually all of the cells present were recovered in the non-adherent fraction. Total progenitor numbers in cultures without feeders were consistently lower than in cultures with feeders, although readily detectable numbers of progenitors usually persisted for several weeks.

From the data shown in Tables 10 and 11 it can be seen that all types of progenitors in these CML PBL cultures were actively dividing regardless of their differentiation lineage, proliferative potential, location in the

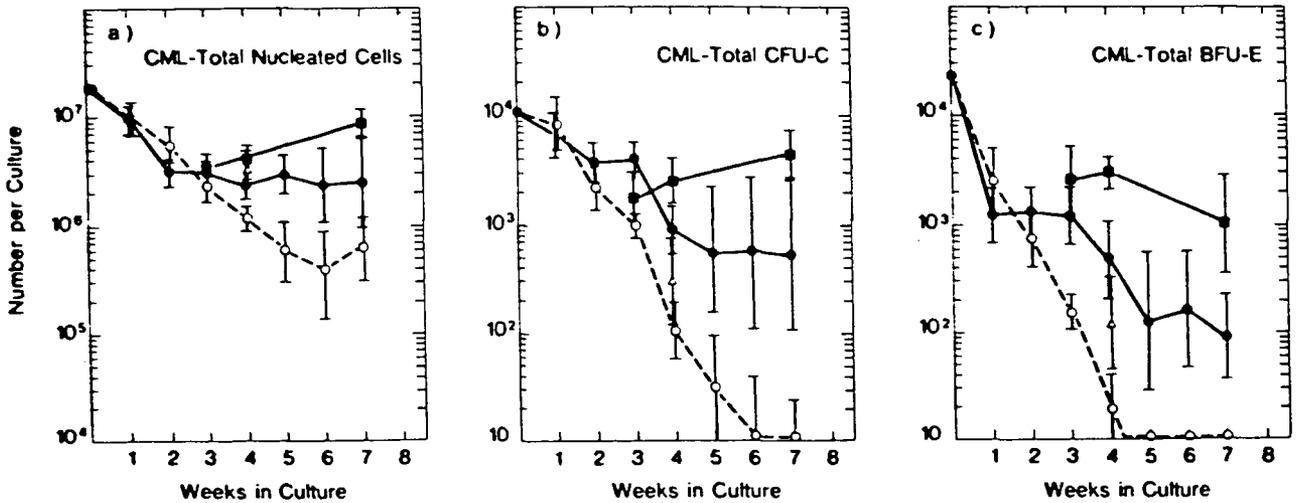


Figure 24. Comparison of total cell and progenitor content of long-term CML PBL cultures initiated with (solid symbols) or without (open symbols) a pre-established normal marrow feeder. Circles - data for non-adherent fractions assessed weekly. Squares - data for adherent fractions (cultures with feeders only, $<10^4$ adherent cells and no adherent progenitors were detected in cultures without feeders). Triangles - data for cultures established without feeders, but from which no cells were removed at each weekly medium change until the culture was sacrificed. Values shown are the geometric means \pm 1 SEM from data of 11 experiments.

Table 10. Thymidine suicide measurements (% kill) of nonadherent progenitors in CML blood cultures

| Feeders | Age of culture | Exp. no. | BFU-E* | | CFU-C† | | |
|---------------------|-----------------------|-----------|-----------|--------|-----------|--------|----|
| | | | Primitive | Mature | Primitive | Mature | |
| Present | 3-4 weeks | 1 | 60 | 45 | 46 | 62 | |
| | | 2 | 69 | 38 | -‡ | 60 | |
| | | 3 | 46 | 69 | - | 42 | |
| | | 4 | 56 | - | - | 47 | |
| | | 5 | 55 | 62 | - | 47 | |
| | | 6 | 52 | 58 | - | 39 | |
| | | 7 | 35 | 50 | - | 59 | |
| | | 8 | 43 | 37 | - | 57 | |
| | 3-4 weeks + 2 days | 2 | - | - | - | 42 | |
| | | 3 | 52 | 75 | - | 50 | |
| | | 4 | 54 | - | - | 54 | |
| | 7 weeks | 1 | 61 | 54 | - | 42 | |
| | | 3 | - | - | - | 37 | |
| | Absent | 3-4 weeks | 1 | 43 | 46 | - | 47 |
| | | | 2 | 44 | 33 | - | 51 |
| 3 | | | 28 | 51 | - | 44 | |
| 4 | | | - | - | - | 55 | |
| 9 | | | 51 | 45 | 63 | 58 | |
| 10 | | | 58 | 52 | - | 51 | |
| 4 weeks + 2 days | | 1 | 43 | 57 | 45 | 42 | |
| | | 4 | - | - | - | 55 | |
| 7 weeks | | 2 | - | - | - | 55 | |

* Primitive - >8 clusters; Mature - 3-8 clusters.

† Primitive - >500 cells; Mature - 20-500 cells.

‡ No data.

Table 11. Thymidine suicide measurements (% kill) of adherent progenitors in CML blood cultures

| Age of culture | Exp. no. | BFU-E* | | CFU-C† | |
|-----------------------|----------|-----------|--------|-----------|--------|
| | | Primitive | Mature | Primitive | Mature |
| 3-4 weeks | 1 | 39 | 50 | 46 | 59 |
| | 2 | 50 | 39 | 44 | 41 |
| | 3 | 32 | 51 | - | 42 |
| | 4 | 49 | 63 | 65 | 45 |
| | 5 | 48 | 46 | 43 | 49 |
| | 6 | 54 | 42 | 64 | 56 |
| | 7 | 58 | 56 | 70 | 57 |
| | 8 | 44 | 52 | 53 | 49 |
| | 9 | 44 | 44 | 52 | 53 |
| | 10 | 75 | 69 | 73 | 62 |
| | 11 | 36 | 44 | 59 | 42 |
| 3-4 weeks + 2 days | 2 | 52 | 42 | 51 | 52 |
| | 3 | 51 | 55 | - | 42 |
| | 4 | 41 | 49 | 65 | 45 |
| | 5 | 48 | 49 | 58 | 52 |
| | 6 | 52 | 50 | 50 | 53 |
| | 7 | 49 | - | 46 | 42 |
| | 7 weeks | 1 | 36 | 57 | - |
| 2 | | 40 | - | - | 43 |
| 7 weeks + 2 days | 3 | 42 | - | - | 42 |

* Primitive - >8 clusters; Mature - 3-8 clusters.

+ Primitive - >500 cells; Mature - 20-500 cells.

adherent or non-adherent fraction, time of assessment after feeding the cultures, or presence or absence of a pre-established feeder. These findings corroborate those described above for the one conventional long-term CML marrow culture where Ph¹-positive progenitor yields were sufficient to allow the cycling behaviour of neoplastic progenitors to be evaluated. However, these results contrast markedly with the findings for normal primitive progenitor types maintained in conventional long-term marrow cultures (Chapter IV).

Colonies generated in assays of cells from both types of CML PBL cultures were also cytogenetically analyzed. The results are summarized in Table 12. These show that the majority of the progenitors in all cultures were Ph¹-positive at the time cycling measurements were undertaken.

D) Cultures of normal PBL on normal marrow adherent layers

Because the difference in primitive Ph¹-positive progenitor cell cycling noted in this study was revealed in a somewhat different system (i.e. CML PBL added to normal marrow adherent layers), a final series of experiments were undertaken to evaluate the cycling behaviour of normal peripheral PBL progenitors maintained under similar conditions.

Weekly changes in the total number of nucleated cells and clonogenic erythroid and granulopoietic precursors detected in normal PBL cultures set up with and without feeders are shown in Figure 25. Results were very similar to those obtained with CML cells, although the initial inoculum of 2×10^7 light density T-depleted normal PBL cells contained 10-100 fewer progenitors, and progenitor numbers measured after 3-7 weeks were correspondingly lower. Nevertheless, adherent layer values, where the majority of the progenitors were again found, were sufficient to allow cycling data to be obtained. The results, shown in Table 13, reveal the same

Table 12. Cytogenetic analysis of CML peripheral blood cultures with and without feeder layers*

| Age of Culture | Patient | With feeders | | Without feeders | |
|----------------|---------|-----------------|-----------------|-----------------|-----------------|
| | | Ph ⁺ | Ph ⁻ | Ph ⁺ | Ph ⁻ |
| 3-5 wks | 1 | 38 | 1 | 1 | 7 |
| | 2 | 87 | 0 | 5 | 0 |
| | 3 | 21 | 0 | 21 | 0 |
| | 4 | 6 | 8 | 45 | 11 |
| | 5 | 35 | 0 | 8 | 0 |
| | 6 | 33 | 0 | 25 | 0 |
| | 7 | 26 | 0 | 9 | 0 |
| 6-10 wks | 1 | 15 | 0 | 1 | 2 |
| | 2 | 14 | 0 | - ⁺ | - |
| | 3 | - | - | - | - |
| | 4 | - | - | - | - |
| | 5 | 21 | 0 | - | - |
| | 6 | - | - | - | - |
| | 7 | - | - | 0 | 1 |

* Values shown are total metaphases obtained from BFU-E and CFU-C colonies cultured in methylcellulose assays.

+ No data.

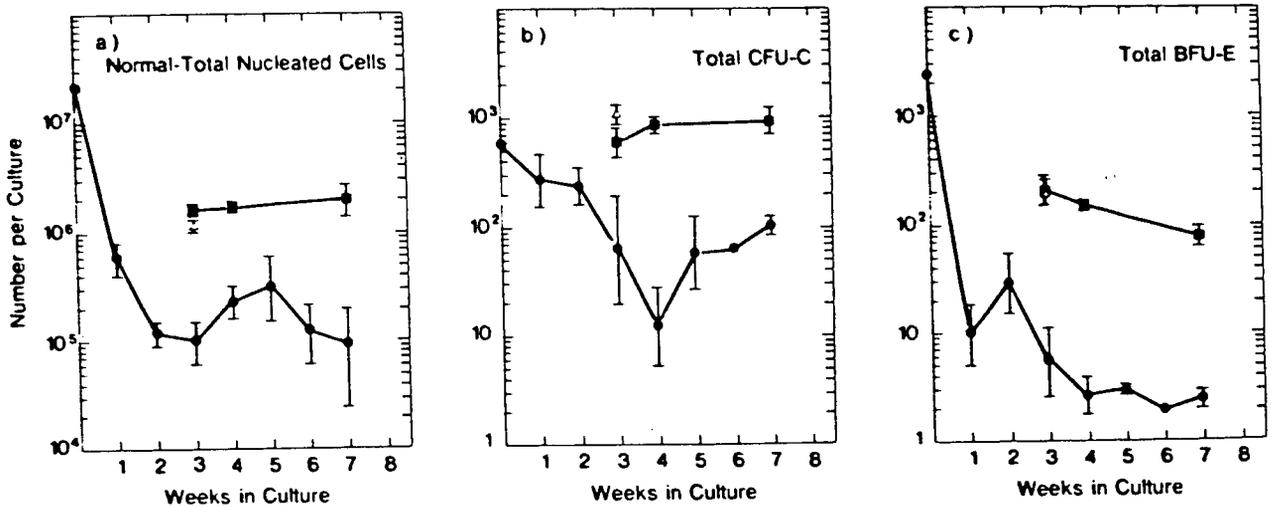


Figure 25. Comparison of total cell and progenitor content of long-term normal PBL cultures initiated with (solid symbols) or without (open triangles) a pre-established normal marrow feeder. Circles - data for non-adherent fractions of cultures with feeders assessed weekly. Squares - data for adherent fractions of cultures with feeders. Triangles - data for non-adherent fractions of cultures without feeders (no adherent fraction obtained), from which no cells were removed at each weekly medium change until the culture was sacrificed. Values shown are the geometric means ± 1 SEM from data of 5 experiments.

Table 13. Thymidine suicide measurements (% kill) of hemopoietic progenitors from the adherent fraction of normal blood cultures

| Feeders | Age of culture | Exp. no. | BFU-E* | | CFU-C† | | |
|---------------------|-----------------------|-----------|-----------|--------|-----------|--------|----|
| | | | Primitive | Mature | Primitive | Mature | |
| Present | 3-4 weeks | 1 | 0 | 50 | 18 | 53 | |
| | | 2 | 0 | 41 | 5 | 48 | |
| | | 3 | 2 | 53 | 0 | 45 | |
| | | 4a | 0 | 40 | 0 | 42 | |
| | | 4b | 9 | 46 | 11 | 44 | |
| | | 5 | 1 | 54 | 0 | 43 | |
| | 3-4 weeks + 2 days | 1 | 41 | 72 | 59 | 57 | |
| | | 2 | 47 | 50 | 48 | 46 | |
| | | 3 | 54 | 60 | 50 | 51 | |
| | | 4 | 49 | 33 | 40 | 43 | |
| | | 5 | 56 | 59 | 53 | 42 | |
| | 7 weeks | 1 | 0 | 43 | 0 | 38 | |
| | | 2 | 4 | 33 | ‡ | 41 | |
| | Absent | 3-4 weeks | 3 | 44 | 46 | - | 54 |
| | | | 4 | 57 | 50 | 62 | 53 |
| 5 | | | 56 | 70 | 52 | 48 | |
| 3 weeks + 2 days | | 4 | 58 | 46 | - | 59 | |

* Primitive - >8 clusters; Mature - 3-8 clusters.

† Primitive - >500 cells; Mature - 20-500 cells.

‡ No data.

pattern of alternating proliferation and quiescence, characteristic of primitive (high proliferative potential) but not mature (low proliferative potential) progenitor cell types located in the adherent layer. In cultures without feeders, the number of progenitors had generally decreased by 3 weeks to values below those measured in cultures with feeders, even when no cells were removed at each weekly medium change (Figure 25). However, at 3 weeks they were present in high enough numbers for cycling measurements to be performed. Interestingly, the results of these showed that in the absence of a feeder, all progenitor types were proliferating continuously, irrespective of the time since the previous medium change (Table 13).

3) DISCUSSION

Currently, there is much interest in defining the mechanisms that regulate pluripotent hemopoietic stem cell turnover. In the murine system, conclusive evidence of direct acting soluble factors that support both self-renewal and differentiative divisions of pluripotent cells in vitro has now been obtained (Johnson et al, 1977; Metcalf et al, 1980; Metcalf and Nicola, 1983). Although similar studies with purified factors have yet to be performed with human cells, recent purification and gene cloning studies (Metcalf, 1985; Welte et al, 1985; Kaushansky et al, 1985) indicate a strong analogy between these species. Thus it may be anticipated that soluble factors capable of activating or contributing to the direct activation of human pluripotent stem cells will be found. On the other hand, studies of the genetically determined Sl/Sl^d anemic mouse indicate that primitive hemopoietic cell proliferation in vivo is dependent on close range interactions with cells that normally do not circulate and do not appear to be derived from hemopoietic progenitors (McCulloch et al, 1965).

Demonstration of localized effects of radiation on stem cell activation in partially shielded mice provide additional support for a non-humoral component to normal stem cell regulation (Gidali et al, 1972).

More recently, evidence for both positive and negative regulation of primitive hemopoietic cell turnover in vitro has been obtained by time course studies of murine (Toksoz et al, 1980) and human long-term marrow cultures (Chapter IV). In the present study we have shown that after activation in culture primitive normal cells of PBL origin are rendered quiescent in the presence of an adherent marrow population, but not in its absence. This indicates that maintenance of primitive hemopoietic progenitor quiescence is a function of an adherent cell unique to the marrow and not present in, or derived from, cell types circulating in the PBL. The present studies have further revealed that primitive Ph¹-positive progenitors from CML patients are not sensitive to this negative control mechanism in vitro. The fact that CML cells remained in cycle despite their maintenance in higher numbers than in those typical of cultures initiated with normal blood, provides additional evidence that the arrest of normal primitive cell proliferation is not due simply to nutrient exhaustion (Chapter IV). In the CML experiments, the lack of cycling control would not appear to be explained by a faulty "stroma", since normal marrow adherent layers were used as feeders. It therefore seems likely that the abnormal behaviour observed is due to a change in the CML cells themselves.

What the nature of this change may be is not clear, although two possibilities invite consideration. One is based on the observation that primitive CML progenitor cells may have altered cell surfaces (Baker et al, 1985). This could lead to a reduced ability to bind to surface determinants on adherent marrow cells that may be an essential aspect of the negative

regulation process. The second, not exclusive, possibility is that primitive CML progenitor cells may have autocrine or autostimulatory capabilities. Such a property might then allow these cells to override a negative signal or replace a transient positive signal, resulting in the phenotype observed.

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CHAPTER VII

CONCLUSIONS AND FUTURE DIRECTIONS

Although clonal assay systems have permitted elucidation of a number of abnormalities characteristic of MPD progenitors, the contribution of these to the pathogenesis of the MPD is unclear. A major limitation of clonal assay systems is their inadequacy to measure pluripotent stem cell self-renewal and their failure to reproduce close range regulatory mechanisms believed to operate in vivo. By their very nature clonal assays minimize direct interactions between cells. Thus, some regulatory factors which may be important in vivo could be totally absent from clonal culture systems.

A growing appreciation of the importance of hemopoietic:stromal cell interactions in in vivo hemopoiesis has led to increased interest in the application of the long term culture system in the study of normal and neoplastic hemopoiesis. Long term cultures provide a complex in vitro system in which hemopoietic progenitors of all myeloid cell lines can be maintained for periods in excess of 8 weeks. Such a culture system permits interactions between various hemopoietic and non-hemopoietic cell types that may closely approximate conditions occurring in vivo. Whether such accessory cell populations are an appropriate model of the intramedullary environment and whether they are able to produce factors that regulate normal stem cell turnover are areas of current investigation, addressed in part by the work described.

The primary objective of this thesis was to characterize possible disease-related alterations in the cycling behaviour of normally quiescent hemopoietic cells in patients with various MPD and to develop an in vitro

model where the biology underlying such alterations might be analyzed further. I therefore first undertook a comprehensive study of the cycling characteristics of primitive and mature progenitors of the erythroid and granulocytic lineages, as well as pluripotent progenitors, in the blood and marrow of patients with PV, ET, and CML. These studies were then extended in vitro by appropriate application or modification of the long-term marrow culture system.

Analysis of cell cycle control in MPD patients

Peripheral blood cells from 9 normal individuals and from 12 SE, 14 PV, and 16 CML patients were examined for the presence of S-phase cells by short term exposure to ^3H -thymidine. All hemopoietic progenitors from normal blood were found to be quiescent, regardless of cell lineage or stage of differentiation, while the majority of such progenitors from the peripheral blood of patients with CML or PV were in S-phase. Marrow cells from 20 normal individuals and from 15 SE, 16 PV, and 13 CML patients were subjected to the same procedure for analysis for their cell cycle activity. In normal marrow stage-specific increases in proliferative activity occur with progressive progenitor cell maturation in both lineages studied. In contrast this progression was absent in PV or CML patients, where all marrow progenitors including the most primitive erythropoietic and granulopoietic compartments were found to be in cycle. The majority of these are quiescent in normal marrow. Such alterations in cycling behaviour were also found to be typical of ET patients in whom erythroid progenitors capable of Epi-independent erythropoiesis could usually also be demonstrated.

This series of experiments indicates that neoplastic hemopoietic progenitor cells from all classes of MPD patients are capable, in some manner, of bypassing normal extrinsic regulatory control. In order to

further characterize the cycling abnormalities observed in the MPD, experiments were undertaken to determine if such abnormalities are also characteristic of neoplastic progenitors produced in the long-term marrow culture system. To obtain control data, long term cultures were established with cells from normal individuals and the proliferative behaviour of progenitors present in these cultures 3-8 weeks later was examined

Normal long term cultures

A total of 82 long-term cultures were initiated with marrow from 15 normal individuals. Progenitors of large erythroid colonies (>8 erythroblast clusters) present in the nonadherent fraction, and progenitors of small granulocyte/macrophage colonies (<500 cells) present in both the nonadherent and adherent fractions were found to be actively cycling at all times examined. In contrast, progenitors of large granulocyte/macrophage colonies (>500 cells) and progenitors of large erythroid colonies (>8 erythroblast clusters), present in the adherent layer, consistently alternated between a quiescent state at the time of each weekly medium change, and a proliferating state 2-3 days later. Additional experiments revealed that the activation of primitive progenitors in the adherent layer was not dependent on the addition of fresh glutamine or hydrocortisone, nor on the physical manipulations involved in changing the growth medium.

These studies provide the first direct evidence that normal long-term human marrow cultures support the continued turnover of a variety of early hemopoietic progenitor cell types. In addition, they indicate that the proliferative activity of the most primitive of these progenitors is normally regulated in vitro by stage-specific cell-cell interactions, that are subject to extrinsic manipulation (i.e. feeding). I then proceeded to analyze the cycling behaviour of progenitors present in long-term MPD cultures.

PV Long-Term Cultures

PV Long-Term Cultures

Marrow cells from 7 PV patients were used to establish long-term cultures. The ability of these cultures to maintain the abnormal clone in vitro was examined using Ep-independence as a marker of neoplastic erythropoietic cells. The proportion of erythroid progenitors exhibiting this phenotype was found to remain unchanged in standard long-term cultures for periods of up to 7 weeks.

The cell cycle status of hemopoietic progenitors in these cultures was also examined. All classes of hemopoietic progenitors were found to be in active cell cycle, regardless of the age of the culture, time after the weekly medium change, progenitor proliferative capacity, or progenitor location (adherent versus nonadherent fraction). Thus, the pattern of regulated proliferation observed in normal long-term marrow cultures was not reproduced in PV cultures. These differences suggest that neoplastic progenitors in PV do not respond to negative influences of regulatory adherent elements that suppress the proliferative activity of their normal counterparts.

CML Long-Term Cultures

Initial experiments with CML cells were concerned with achieving some maintenance of Ph¹-positive progenitors under long-term culture conditions. The effect of seeding CML cells on irradiated normal marrow adherent layers was evaluated with marrow specimens from 4 patients. Cytogenetic analysis and the assessment of progenitor numbers revealed that such modifications of the culture system resulted in a limited improvement in the maintenance of Ph¹ progenitors. As a next step, peripheral blood cells from CML patients were layered over irradiated normal adherent layers. Eleven such experiments were undertaken, using cells from 7 CML patients. Under these conditions CML

PBL progenitors could be maintained for periods of 1-2 months. Progenitor numbers were found to be higher in the adherent layer than in the nonadherent fraction, and the number of Ph¹-positive primitive progenitors of high proliferative potential present in the adherent layer was sufficient for determinations of their cycling status. Such measurements demonstrated that primitive CML progenitors cycle continuously regardless of the presence or absence of an adherent feeder layer. Similar results were obtained with marrow cells from one anomalous CML patient whose Ph¹-positive progenitors could be maintained in standard long-term marrow cultures.

In contrast, primitive PBL normal progenitors, when cultured in the presence of a pre-established adherent marrow feeder layer were found to go in and out of cycle after each medium change. In the absence of a feeder layer they remained continuously in cycle. In both CML and normal PBL cultures, low proliferative potential progenitors were in constant cell cycle whether or not a feeder layer was present.

This series of long-term culture experiments extends the observations obtained with the in vitro studies of PV marrow cells and demonstrates that in CML, as in PV, the abnormalities in cell cycle behaviour seen in vivo in the MPD can be reproduced in vitro.

Conclusions

Two significant findings emerge from this work:

- 1) Regulation of normal primitive progenitor proliferation in long-term cultures is determined by close range interactions with a cell type(s) derived from and unique to the marrow. These interactions may be directly between cells, or may involve short range regulatory factors. An important function of these adherent cells is to return and maintain primitive progenitors in a quiescent state.

2) Primitive progenitor cells in at least 3 of the MPD demonstrate a common abnormality in the control of primitive progenitor cell turnover in vivo. Studies with the long-term cultures indicate that such alterations in cycling behaviour may be due to an intrinsic fault in the neoplastic stem cells themselves, which permits them to override or ignore negative regulation imposed by normal adherent marrow elements. Such insensitivity to negative regulation may be explained either by the activation within MPD progenitors of mechanisms for autostimulation and/or mechanisms that impair negative signal recognition or transduction.

Future Directions

Although data presented in this thesis supports the concept that alterations in cycling behaviour may be involved in the pathogenesis of the MPD, a number of questions remain unanswered. The absence of lineage specific alterations in cell cycle activity indicates that other mechanisms must be operative to produce the typical clinical presentation of PV, ET, or CML. The possibility that abnormalities in cycling behaviour and the expression of Ep-independence may have a common mechanistic basis remains to be explored. In addition, the nature of the lesion in the abnormal stem cell that permits its progeny to bypass normal regulatory controls has not yet been determined.

Long term cultures form a complex in vitro system that has not been fully characterized. Regulatory influences, stimulatory or inhibitory, may have numerous sources. Further work is needed to examine the components of the system, and to identify those involved in stem cell regulation. One possible approach is to isolate pure populations of adherent marrow cells and determine the ability of such cells to maintain and regulate hemopoiesis. Another approach is to undertake biochemical analysis of spent

growth medium from such cells to identify and characterize factors that may be released by them.

Finally, one must consider the ultimate goal of all medical research - the application of new information to the treatment, and perhaps the cure, of disease. If abnormalities in cycling behaviour are a significant factor in the pathogenesis of the MPD, then elucidation of the underlying mechanism may eventually lead to the development of techniques by which hemopoietic cells in these diseases could be made responsive to normal regulatory influences.

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